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THE ROLE OF MACROPHAGES IN REGULATING INFLAMMATION BY OXIDATIVE BURST

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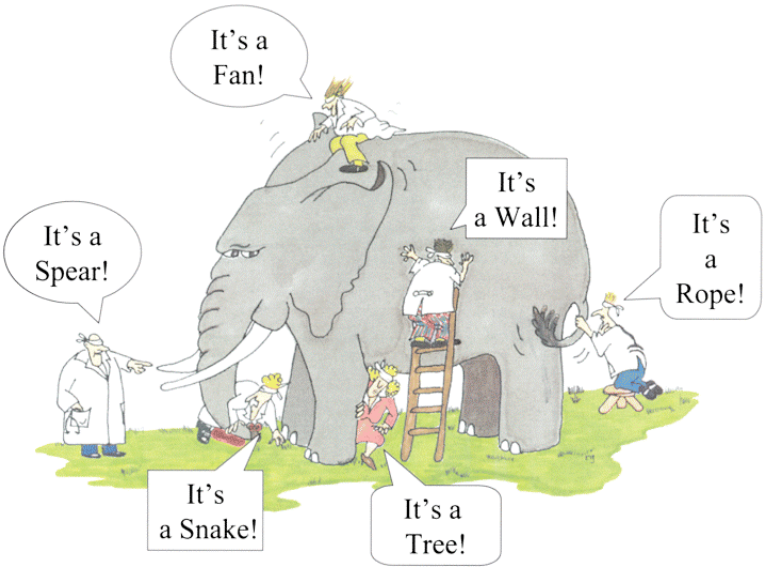
To my past and present colleagues, mice and men



(G.Renee Guzlas in (1))

"Science is the quest for truth. But the truth is not *certain* truth."
(Sir Karl Popper, *All life is problem solving*)

"It is not the possession of knowledge, of irrefutable truth, that makes the scientist,
but the persistent and recklessly critical quest for truth."
(Sir Karl Popper, *The logic of scientific discovery*)

ABSTRACT

The production of reactive oxygen species (ROS) through a process called oxidative burst is an essential defence mechanism against pathogens. In phagocytes, such as neutrophils and macrophages, the NADPH oxidase 2 (NOX2) complex is the main source of ROS. Genetic alterations in any of the components of the NOX2 complex that impair the ROS production are at the origin of a condition called chronic granulomatous disease (CGD), characterized by recurrent life threatening bacterial and fungal infections. Recently, natural occurring mutations in *Ncf1*, a regulatory component of the NOX2 complex, were described to compromise the protein function and to increase arthritis severity in rats and mice. Macrophages are phagocytes that express NCF1 and are able to kill pathogens. At the same time, they are antigen-presenting cells known to play an important role in arthritis. We therefore hypothesized that expression of NCF1 in macrophages would have an impact on the immune response during arthritis and bacterial infections. The aim of the studies presented in this thesis is to evaluate the influence of NCF1 expressed by macrophages on development of arthritis and resolution of bacterial infections.

Using transgenic mouse models we could describe a role for macrophages in both priming and activation of arthritogenic T cells. In a first transgenic mouse, expression of functional NCF1 restricted to macrophages reduced arthritis severity, priming of Th1 T cells and T cell proliferation, therefore limiting the T cell-dependent autoimmune outbreak. In a second transgenic mouse strain, where macrophages were the only cells expressing the arthritis-prone MHC class II A^q molecule, macrophages could prime arthritogenic T cells and mediate arthritis development, but only in NCF1 deficient setting. We could conclude that ROS production by macrophages is important in determining the activation state of T cells and in regulating the severity of arthritis.
As in the human CGD situation, mice carrying the *Ncf1* mutation were more susceptible to spontaneous and induced bacterial infections. Using the transgenic mouse where macrophages expressed the functional NCF1, we observed that macrophage-derived ROS effectively protected mice from bacterial infections, a function believed to be executed mainly by neutrophils.
Finally, we tested a new model of arthritis where the disease was induced with a peptide of a glycolytic enzyme. We found that the symptoms and pathogenesis of the disease resembled the one of the most common arthritis model, collagen-induced arthritis, which is induced with the full collagen protein. Both diseases are dependent on an intact adaptive immune system and their severity is influenced by *Ncf1*. We were also able to identify one of the important residues causing the peptide's arthritogenicity.

In summary, our data highlight the crucial role of NCF1, and consequently of NOX2 complex, in regulating both innate and adaptive immune responses. NCF1-dependent ROS in macrophages was important during both phagocytosis and antigen presentation, resulting in clearance of bacterial infection and suppression of chronic inflammation. These findings will facilitate further investigations of the molecular pathways through which ROS influence arthritis pathogenesis and hopefully lead to identification of new therapeutic targets.

LIST OF PUBLICATIONS

I. ***Macrophages suppress T cell responses and arthritis development in mice by producing reactive oxygen species.***
Kyra A. Gelderman, Malin Hultqvist, Angela Pizzolla, Ming Zhao, Kutty Selva Nandakumar, Ragnar Mattsson, Rikard Holmdahl.
Journal of Clinical Investigation 2007; 117(10):3020-8

II. ***CD68-expressing cells can prime T cells and initiate autoimmune arthritis in the absence of reactive oxygen species.***
Angela Pizzolla*, Kyra A. Gelderman*, Malin Hultqvist, Mikael Vestberg, Kenth Gustafsson, Ragnar Mattsson, Rikard Holmdahl.
European Journal of Immunology 2011; 41(2):403-12

III. ***Reactive Oxygen Species produced by the NOX2 Complex in Monocytes Protect from Bacterial Infections in Mice.***
Angela Pizzolla, Malin Hultqvist, Bo Nilson, Melissa J. Grimm, Tove Eneljung, Ing-Marie Jonsson, Margareta Verdrengh, Tiina Kelkka, Inger Gjertsson, Brahm H. Segal, Rikard Holmdahl.
Submitted

IV. ***A new model of arthritis induced by a glucose-6-phosphate isomerase peptide: immunological requirements and peptide characterization.***
Angela Pizzolla, Frida Laulund and Rikard Holmdahl
Manuscript

* These authors contributed equally to the work

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LIST OF ABBREVIATIONS

ACPA	Anti-citrullinated protein antibody
APC	Antigen presenting cell
CAIA	Collagen antibody induced arthritis
CD	Cluster of differentiation
CFA	Complete Freund's adjuvant
CGD	Chronic granulomatous disease
CIA	Collagen-induced arthritis
CII	Type II collagen
CTLA-4	Cytotoxic T lymphocyte associated
DC	Dendritic cell
DMARM	Disease modifying antirheumatic drug
EAE	Experimental autoimmune
fMLF	formyl-methionyl-leucyl-phenylalanine
GCs	Glucocorticoids
GPI	Glucose-6-phosphate isomerase
HLA	Human leukocyte antigen
IFN-γ	Interferon-gamma
IgG	Immunoglobulin G
IL	Interleukine
LAT	Linker of activation of T cells
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
MS	Multiple sclerosis
NADPH	Nicotinamide adenine dinucleotide
NCF	Neutrophil cytosolic factor
NOX2	NADPH oxidase 2
NSAIDs	Nonsteroidal anti-inflammatory drugs
Phox	Phagocyte oxidase
PMA	Phorbol-12-myristate-13-acetate
RA	Rheumatoid arthritis
RF	Rheumatoid factor
ROS	Reactive oxygen species
SE	Shared epitope
SNP	Single nucleotide polymorphism
TCR	T cell receptor
Th	T helper cell
TNF-α	Tumor necrosis factor alpha

THE IMMUNE SYSTEM IN A NUTSHELL

The immune system has the vital task to protect the body from external and internal dangers that threaten the health and functionality of the organism. All pathogens (viruses, bacteria, yeasts and worms) that come from outside the body are considered external dangers. Internal dangers are malignant cells that develop during cancer. Internal and external dangers share the capacity to colonize the body and to utilize its resources in order to expand, ultimately leading to the death of the host organism. The immune system has evolved several strategies to fight threats that come in very different sizes and shapes and that colonize different compartments of the body. The first line of defence against intruders is the physical barrier provided by the skin and the mucous membranes, which separate the body from the environment and are equipped with antimicrobial compounds. Just behind these doors, an efficient and fast machinery is waiting, ready to shoot, to kill pathogens and remove infected cells: the **innate immune system**. The innate immune system is so called because it does not change during the lifetime and provides non-specific protection. Innate immunity comprises different types of cells: the phagocytes (literally: eating cells) that eat up pathogens; the natural killer cells that directly kill infected cells; mast cells, basophils and eosinophils that release toxic substances; a set of molecules, the complement system, that perforate or tag an invader. One of the weapons used by the innate immune cells to kill pathogens is reactive oxygen species (ROS). These derivatives of oxygen react very quickly with biological molecules and irreversibly damage them. ROS are produced mainly by two types of phagocytes, neutrophils and monocytes. Defects in the production of ROS lead to recurrent and life threatening infection and chronic inflammation: a disease called chronic granulomatous disease (CGD) (2). In paper III of this thesis it will be shown that monocytes alone can protect the organism from bacterial infections by producing ROS.

Often the action of the innate immune system alone is not sufficient to eliminate the threat, therefore another system, the **adaptive immune system,** gets called to arms. The adaptive immune system confers specific protection, tailor-made for the characteristics of the pathogen. Two cell types constitute the adaptive immune system, T cells and B cells, also called lymphocytes. They share the capacity of randomly modifying genes, via rearrangements and mutations, in order to create unique molecules that can bind a vast number of pathogens never encountered before in the life of the host. Two subtypes of phagocytes, namely macrophages and dendritic cells, activate T cells by presenting them pieces of eaten pathogens called antigens. Antigens are bound to special molecules called major histocompatibility complex (MHC) on the surface of phagocytes forming antigen-MHC complex. Activated T cells can either kill the infected cells directly or help B cells to produce antibodies, which will then assist the innate immune system in performing its task. After activation, memory T cells and B cells remain in the host for the rest of its life and are quickly reactivated upon re-infection. The activation of the innate and adaptive immune system leads to inflammation that needs to be limited in space and time not to permanently damage the host.

Due to the random generation of molecules that recognize the antigen-MHC complex, T cells have to be stringently selected to recognize the MHC of the host, but only in combination with foreign antigens and not in combination with pieces of proteins from

the host (autoantigens). This selection is not completely stringent, so cells that can recognize autoantigens are present in the body. These cells are called autoreactive. Several mechanisms make sure that autoreactive cells are eliminated or silenced in a process called tolerization. Nevertheless, it is still possible that some autoreactive T cells and B cells escape all the checkpoints and get activated. Then **autoimmunity** arises, a condition where the body attacks itself. About 5% of the world population suffers from autoimmune diseases, some of which are limited to one organ, as multiple sclerosis, diabetes or thyroiditis, while some others are systemic as systemic lupus erythematosus or rheumatoid arthritis (RA) (3).

RA symptoms can vary and it is considered a syndrome more than a single disease. It is characterized by chronic inflammation in the joints and affects about 1% of the population (4). RA is a complex disease where genetic and environmental factors interact, making it difficult to identify exactly which factors have an impact on the disease development just by studying the heterogeneous human situation. Therefore the use of animal models, where the environment and/or the genetic background can be tightly regulated, allowed remarkable advances in the study of RA. Using a rat models for RA, a gene that regulates arthritis severity has been identified (5). The gene is *Ncf1*, a regulatory component of the NADPH oxidase complex, which produces ROS in phagocytes. The most renowned function of ROS is its antimicrobial activity but it is also known as a signalling molecule. Recently more and more evidence has shown a role of ROS in regulating the adaptive immune response. After the role of *Ncf1* was confirmed in a mouse model of arthritis (6), the aim is now to understand which pathways are affected by this gene. In this thesis, three papers focus on investigating the role of *Ncf1* in activation of T cells, a crucial cell type in arthritis, using different mouse models of the disease.

RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is a chronic inflammatory disease affecting peripheral joints, most commonly hands, feet and elbows. It is characterized by joint swelling and pain that eventually lead to destruction of the tissue and functional disability. RA is associated with lower life expectancy and high risk of developing other disorders (comorbidity), especially cardiovascular diseases and other autoimmune diseases (3, 4, 7). Women are more frequently affected than males and the overall prevalence on the total population is about 1% in developed countries (4). The disease can manifest at any age with a peak of onset at 50 years of age. It is considered to be an **autoimmune disease** due to the presence of T cells and B cells specific for autoantigens, like type II collagen (CII) and citrullinated proteins (8-12). The B cell response is best characterized for its production of autoantibodies as anti-IgG called rheumatoid factor (RF) and anti-citrullinated proteins antibodies (ACPAs) that arise years before disease manifestation (13-15). As the symptoms of RA are similar to other inflammatory arthritidies a set of criteria have been established to ensure correct diagnosis (16). These criteria have recently been updated by the American College of Rheumatology and the European League Against Rheumatism (17). They take into account the number of joints affected, the presence of RF or ACPAs, acute phase reactants and the duration of the symptoms. The criteria are intended to identify RA at an early stage to be able to intervene with proper treatment as early as possible and break the disease progression. No cure for RA is available so far.

DISEASE PATHOLOGY

The exact pathological mechanisms that drive RA are still unknown. Some insights have been gained from comparing the cellular and molecular components of inflamed joints with healthy ones. The most affected joints in RA are synovial joints, as knees, fingers and hips. They constitute the junction between two or more bones and are characterized by a space between the bones, called synovial cavity, filled with synovial fluid. The junction of the two bones is surrounded by the articular capsule, which seals the synovial fluid from the surrounding tissue. The inner layer of the articular capsule is called synovial membrane (or **synovium**). It secretes the synovial fluid and supplies nutrients to the cartilage that covers the bones. During RA, inflammation of the synovium occurs (synovitis). The macrophage-like and fibroblast-like cells that compose the synovium proliferate and produce excess of synovial fluid together with pro-inflammatory cytokines, chemokines, matrix metalloproteinases, growth factors and even complement proteins and RF (18, 19). All these soluble components actively destroy the extracellular matrix by sustaining the proliferation and activation of the synovial cells and recruiting new immune cells from the blood stream.

Both adaptive and innate immune cells are found in the RA synovium. The presence of activated and memory CD4+ **T cells** in the synovium, typical of RA (20), agrees with the association of the *HLA-DRB1* gene to RA, which points towards the MHC class II activation of T cells being a pathogenic mechanism in RA. Several antigen-presenting cells (APCs) are present, including B cells, dendritic cells, and macrophages that sample the tissue antigens and present them to T cells in lymph nodes (21). The formation of ectopic lymphoid follicle-like structures with germinal centers in the

synovium is characteristic of RA and may be important in sustaining a local inflammation and B cell activation (22). **B cells** producing autoantibodies have been found in the synovium (23). **Innate immune cells** such as neutrophils and mast cells are also recruited to the inflamed synovium and produce cytokines, proteases and ROS that collaborate to the inflammatory milieu and to the cartilage destruction. High ROS levels have been detected in inflamed tissue and can contribute to the tissue damage (reviewed in (24)). New blood vessels are created in the normally avascular synovium, a process called neovascularization. Sustained inflammation leads to the formation of a tumor-like structure called pannus, formed by **macrophages**, osteoclasts (cells that degrade bone) and fibroblast-like synoviocytes, which destroy bone and cartilage. This massive infiltration of cells and fluids causes swelling, pain and erythema. Cartilage, a thin layer of extracellular matrix with covers the bones and dampens friction between bones, is also infiltrated by immune cells, which destroy and erode the tissue leading to permanent joint deformation.

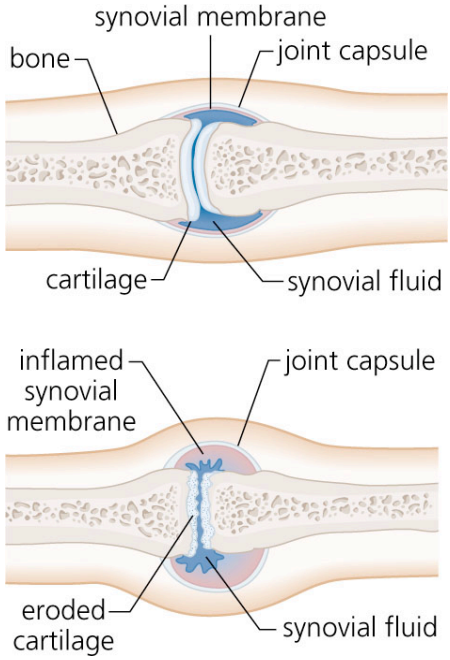

Figure 1 Illustrative picture of a healthy joint on top and a RA joint on the bottom. Drawing from Universalium 2010

RISK FACTORS

Both genetic and environmental factors influence the development of RA.
Genetic predisposition to RA accounts for about 60% of the liability to disease, as established in studies with twins (25, 26). The first locus to be associated with RA was the Human Leucocyte Antigen (**HLA**) in 1976 (27), which is the human nomenclature for the **MHC**. Until today HLA is the strongest known genetic factor associated with RA. The HLA locus comprises a vast number of genes coding for key molecules in antigen presentation. Several alleles of genes encoding for the beta chain of the HLA-DR molecule confer increased risk of developing RA. The RA-associated alleles were found to share a sequence of five amino acids, called the shared epitope (SE), Gln/Arg-Lys/Arg-Arg-Ala-Ala, in a region of the DRβ chain that contributes to the antigen-

binding site (28). It has been postulated that the SE allows the presentation of arthritogenic peptides to T cells (28).
The HLA locus contributes to about 30% of the total genetic risk (29), leaving room for an influence of other non-HLA genes in the disease. These genes are much harder to identify due to their modest effect and their wide spreading in the population. Almost 30 years later the first non-HLA gene was associated with RA: the peptidylarginine deiminase type 4 (*PADI4*) in 2003, only confirmed in the Japanese population (30). *PADI4* encodes for the enzymes that modify arginine into citrulline, the target of the ACPA autoantibodies. One year later another gene was described as a risk factor for RA: the protein tyrosine phosphatase non-receptor, type 22 (*PTPN22*) (31). A single nucleotide polymorphism (SNP) in *PTPN22* is associated with several autoimmune diseases, including RA. This SNP decreases the responsiveness of T cells and B cells to antigen stimulation and alters the numbers of memory T cells and memory B cells (32, 33). The SNP is thought to impair the selection of lymphocytes, leading to release of autoreactive cells into the circulation or lower activity of regulatory T cells (32). Thanks to technical advances, several other loci have been associated with RA in the last years. Many of them contain genes already known to affect the immunological responses, as *CTLA4* (34)*, TRAF1/C5* (35)*, STAT4* (36) and *CD40* (37). For most of the associated loci, the causative SNP has not been identified and the current challenge is to clarify the molecular mechanisms linking the genes and the disease pathogenesis.

As RA results from the interaction of genes and environment, some environmental factors that predispose to disease have been identified. The most studied environmental factor is cigarette **smoking**, identified for increasing risk of RA in 1987 and confirmed further in several studies (38). Interestingly an interaction between smoking and the HLA-DRB1 SE alleles has been found to increase the risk of developing ACPA-positive RA (39, 40). Smoking induces citrullination of proteins in the lungs (40) and T cells can recognize citrullinated antigens presented on MHC class II and HLA-DRB1 molecules (Snir et al. ; Ireland et al. 2006). Recently T cells with this specificity have been identified in RA patients (9).
Since long **infectious agents** have been suspected to be one of the causes of RA development, but no conclusive evidence has been shown so far. Immune responses to different pathogens have been observed to correlate with RA, including bacteria, such as *Escherichia coli, Proteus mirabilis* and *Klebsiella pneumoniae,* mycoplasma and viruses, such as Epstein–Barr Virus (EBV) and Cytomegalovirus (reviewed in (41)). Recently periodontitis, an oral infection, has been associated with RA (42). Peridiontitis is often caused by the bacterium *Porphyromonas gingivalis* that is a unique prokaryote with a peculiar capacity to citrullinate human proteins (43). *P. gengivalis* infections can lead to a break in the tolerance to citrullinated antigens and formation of ACPAs also in non-RA patients (41). These findings have promoted the hypothesis that *P. gingivalis* infection may be one of the causes for development of RA (44, 45).
The influence of **hormones** on RA development is suggested by the fact that women are more susceptible than males. Several studies have addressed the influence of hormones or the use of oral contraceptives on the risk of developing RA and they showed contrasting results, even though most of them conclude that there is no association between the use of oral contraceptives and arthritis (38, 46). There is little consensus also on the effect of parity and breast-feeding on RA development (47-49).

Diet is an important environmental factor. Alcohol consumption was found to decrease the risk of developing arthritis in Scandinavian cohorts (50) while meat consumption seemed not to alter the risk (51). Dietary anti-oxidatidants have been shown to be preventive but not to improve established disease (52).
A lower socioeconomic status, measured by education and occupational class, has been associated with a higher risk of developing RA in Scandinavian countries (53, 54).

In conclusion, in RA, the most influential factors associated with the disease have been identified about 30 years ago and confirmed by several studies, such as the HLA locus and smoking. A number of other genes and environmental factors that contribute for a small percentage to the total risk have been recently identified and are under investigation.

TREATMENTS

Although no cure is available for RA, a wide spectrum of treatments is nowadays in use with the main goal of alleviating the symptoms and slowing down disease progression. Pharmacological treatments are the most used intervention and will be discussed below. Non-pharmacological treatments include physical activity (55) and surgical removal of inflamed synovium (56).
Pharmacological treatment can be divided into three classes of drugs according to their mechanism of action.
Anti-inflammatory drugs and analgesics reduce the pain and the inflammation promptly. The anti-inflammatory drugs can be divided into glucocorticoids and non-steroideal anti-inflammatory drugs (NSAIDs). Glucocorticoids (GCs) are derivatives of natural anti-inflammatory hormones and act as potent anti-inflammatory and immunosuppressants by regulating the expression of several genes. They can slow down disease progression and they are still very extensively used, especially before a definite diagnosis of RA. Their side effects however limit prolonged usage (57). NSAIDs, as aspirin, diclofenac and ibuprofen control pain by inhibiting prostaglandin formation and they are anti-inflammatory but cannot slow disease progression. As pain-relief also medical use of cannabis has been proven efficient (58).
Disease-modifying anti-rheumatic drugs (DMARDs) are the first drugs to be administered after diagnosis of RA. They act slowly but powerfully in reducing cell functions and proliferation thereby affecting the immune system for a long time and inhibiting the disease progression. The most used DMARM is Methotrexate (MTX), which inhibits cell proliferation, T cell activation and induces apoptosis (59, 60). Interestingly the efficiency of this drug is dependent on ROS production *in vitro* (reviewed in (61)).
If a patient fails to respond to DMARDs, newer drugs called **biologic drugs** are prescribed. These comprise monoclonal antibodies and recombinant proteins that specifically target one pro-inflammatory molecule and inhibit or block its action. The first biological drug to be launched was a monoclonal antibody against tumor necrosis factor alpha (TNF-α), a central cytokine in RA, which is now successfully used, often in combination with DMARDs (62). Other monoclonal antibodies that target cytokine pathways have recently been described and proven efficient in the treatment of RA patients: anti-IL1 and IL-6 receptors (63, 64) and anti-IL15 (65). Other biologics directly target adaptive immune cells by depleting them or inhibiting their activation.

This is the case for the anti-B cells therapy that uses the anti-CD20 antibody to remove B cells (66), or the fusion molecule between cytotoxic T-lymphocyte antigen-4 (CTLA-4) and immunoglobulin (Ig) (CTLA-4-Ig), which inhibits T-cell activation (67). As the biologic drugs inhibit the immune system, they have been reported to increase the risk for infectious diseases and cancer. Although a recent meta-analysis did not detect a significant increased risk for these pathologies in the short-term after treatment with biologics, it highlighted a higher risk for adverse events and emphasized the need of long-term safety assessments (68).
As the need for safe and effective drugs is still very present, other therapeutic strategies are under investigation, as well as personalized therapy.

ANIMAL MODELS OF RHEUMATOID ARTHRITIS

As described in the previous chapter, rheumatoid arthritis is a complex disease where several genes and environmental factors interact and contribute to the development of the pathological condition. To develop effective treatments, understanding of the mechanisms that lead to disease is fundamental. Nevertheless, the complexity of the disease, the heterogeneity of the human population, in terms of genetic and environmental variations, and ethical restrictions complicate the human studies enormously. In humans it is almost impossible to study the initial phase of the disease, as it is often asymptomatic and occurs over several years.

To circumvent several of these obstacles, animal models of arthritis are used, most often with mice and rats. Animal models allow control and manipulation of the genetic background and the environment independently, enabling specific studies of the influence of only one or a few elements and the detection of very small effects on the disease. The genetic background is controlled using inbred strains, where all individuals are genetically identical. By crossing susceptible and resistant strains of mice or rats, it has been possible to identify genes that protect from or increase susceptibility to disease, such as *Ncf1* (5). These genes are not possible to pinpoint in humans due to their too small effect or too complex genetic structure. Furthermore, today's technology enables specific alterations at the level of one gene, as for example expression of a foreign gene, called transgene, or inhibition of the expression of one gene, called knock-out. Similarly, the environment can be controlled by housing the rodents in animal houses where food, air, water and pathogen exposure are constant and monitored.

Few strains develop arthritis spontaneously while in most cases the disease needs to be induced. The induction is most commonly achieved by injection of substances that trigger the immune system, as oils, adjuvants, proteins, bacteria, pathogen components or antibodies. Artificial induction allows a time control over the disease and the possibility to intervene at different time points of the disease course, so to further dissect pathological mechanisms. Despite the availability of several animal models of arthritis, each model reflects only some of the kaleidoscopic characteristics of human arthritis.

Figure 2: Typical mice used for experiments. Image: OAK RIDGE NATIONAL LABORATORY

In the next paragraphs, I will describe in details three inducible arthritis models that have been used in the papers presented in this thesis. Other currently used models include the spontaneous arthritis in SKG mice and TNF-α transgenic mice. SKG mice carry a mutation in the *ZAP70* gene (69). ZAP70 acts as a signalling molecule downstream of the TCR (70). The mutation weakens the TCR signal and allows the selection of autoreactive T cells (69). The disease develops spontaneously in conventional animal houses, but in pathogen-free conditions it needs to be induced by injection of yeast extract (71). This observation argues for an infectious component in arthritis development. TNF-α transgenic mice overexpress the human TNF-α molecule (72): as the arthritis develops only in presence of the genetic modification, it is not fully considered as a spontaneous arthritis model.

COLLAGEN INDUCED ARTHRITIS

Type II collagen (CII) is the most abundant protein in the hyaline cartilage, the type of cartilage that surrounds bones in the joints. CII was found to induce arthritis in susceptible strains of rodents when injected intradermally in combination with an adjuvant (73, 74). The model is called collagen-induced arthritis (CIA) and is one of the most used. Disease symptoms appear 3 weeks after immunization with swelling, erythema leading to cartilage and bone erosion (73). One of the important factors in CIA susceptibility is the **MHC class II** region: H-2^q and H-2^r haplotypes confer susceptibility to arthritis induced with heterologous CII, while others confer resistance (75). Within the MHC class II region, the Aβ gene of q haplotype (A^q) confers susceptibility to disease (76). One of the reasons for the susceptibility of the H-2^q strain and the resistance of the H-2^p strain can lye in the higher affinity of the A^q molecule for the immunodominant T cell epitope, the $CII_{256-270}$ peptide, than the A^p molecule (77). Both rat (heterologous) and mouse (autologous) CII can induce arthritis in A^q mice (78). The rat $CII_{256-270}$ peptide differs for one amino acid from the mouse $CII_{256-270}$ peptide and it binds with higher affinity to the A^q molecule(77). This difference in affinity may explain why the rat collagen induces a more severe form of arthritis than the mouse one (78). Both innate and adaptive immune systems are important for CIA development and their cellular components are present in the inflamed joints (79). Among APCs, **macrophages** but not Langherans dendritic cells can present CII to T cells (80). **T cells** and **B cells** are essential to induce CIA (81, 82). Both lymphocytes are activated against CII epitopes, as found in RA patients (8, 10, 11). In A^q expressing mice, the immunodominant T cell epitope has been located between position 256 and 270 of CII (83). To characterize the T cell response, cytokine production was monitored. To prove the importance of cytokines in the disease pathogenesis, cytokines were selectively eliminated from the system with the use of genetically modified mice or antibody depletion. This led to a complex picture, as cytokines may have several redundant roles. A common view today is that Th1 and Th17 cells drive arthritis while Th2 cells suppress the symptoms in the CIA model. In CIA B cells produce arthritogenic antibodies that are directed towards several epitopes of CII (84, 85). Among the innate immunity factors, the **complement system** has been extensively studied and identified as a key player in the disease development (86). Outside the classical immune pathways, *Ncf1* has been identified as a factor that increases arthritis susceptibility and severity in CIA (5, 6). Paper I and II investigate how *Ncf1* influences T cells activation in CIA.

COLLAGEN ANTIBODY INDUCED ARTHRITIS

Arthritis can be induced by injection of serum from arthritic mice or RA patients (84, 87, 88). Monoclonal antibodies specific for CII were derived from CII immunized mice and injected into naïve mice: the recipient mice developed arthritis and the model is then called collagen antibody induced arthritis (CAIA) (85, 89, 90). The onset is rapid and arthritis can be seen after 2 days and it resolves after a month, leaving the mice healthy. LPS increases disease severity and incidence (90, 91). CAIA is independent of MHC class II, T cells and B cells (90, 92), but it requires the presence of neutrophils, complement system and FcRγ-chains (92-94). It is therefore a model where the activation of the **innate immune system** is sufficient to induce disease. The inflammatory response is triggered by deposition of the antibodies on the cartilage, which attracts and activates the complement system, macrophages and neutrophils. It allows studying effector pathways of arthritis without involving the priming phase.

K/BXN MODEL AND GLUCOSE-6-PHOSPHATE ISOMERASE INDUCED ARTHRITIS

The K/BxN model is a spontaneous model of arthritis that arises at around 3 weeks of age in KRN TCR transgenic mice (recognizing a peptide of bovine RNase on A^k) on C57Bl/6 (B6) background, crossed with NOD mice carrying the A^{g7} allele (95). As in CIA, the adaptive immune system is required for arthritis development (95). In the context of A^{g7}, T cells and B cells recognize a ubiquitously expressed glycolytic enzyme, **glucose-6-phosphate isomerase (GPI)** (96). Serum from arthritic mice can transfer disease to naïve mice (97), as well as anti-GPI monoclonal antibodies derived from arthritic animals (98). The K/BxN serum transfer model operates through the innate immune system (complement, neutrophils, mast cells and macrophages) and FcR in a similar fashion to CAIA (99-104).

Immunization with recombinant human (h)GPI protein in complete Freund's adjuvant (CFA) is able to induce arthritis in susceptible strains: DBA/1 or C3H (105, 106). The disease depends on both adaptive and innate immunity: T cells, B cells and the right MHC allele are necessary to induce the disease, as well as FcγR positive cells and monocytes/macrophages (105-108). Antibody treatment directed against IL-17, IL-6, TNF-α or blocking CD28 signalling with CTLA-4-Ig ameliorate the disease (106, 109). T regulatory cells are important to control the chronicity of arthritis. The disease normally resolves about 5 weeks after immunization in DBA/1 mice but it becomes chronic in mice depleted for T regulatory cells (108). Recently a pathogenic role of IFN-γ receptor in this model of arthritis has been described (110).
In 2008 a group in Japan identified a peptide of hGPI that could induce disease in DBA/1 animals, leading to a cross-reactive response to the murine peptide (111). Immunization with the $hGPI_{325-339}$ peptide induces Th1 and Th17 responses (111). However the peptide-induced arthritis is milder than the disease induced by the full protein and immunization with the peptide induced lower titers of anti-hGPI antibodies than with the full protein (111, 112). In paper IV we investigated the arthritis generated by immunization with the $hGPI_{325-339}$ peptide in C57BL/10.Q mice, which share the $H\text{-}2^q$ haplotype with DBA/1 mice.

OXIDATIVE BURST

Oxidative burst (or respiratory burst) is the process of rapid production of **reactive oxygen species (ROS)**. ROS comprise a variety of small molecules, which contain an oxygen atom and are highly reactive with other molecules. The reactivity is due to the oxygen atom being unstable due to lack of electrons and to its strong tendency to acquire the missing electrons from other molecules: a process called oxidation. ROS are therefore strong oxidizing agents or oxidants and can reversibly or irreversibly oxidize almost all biological molecules. The most common ROS, and the enzymes that catalyse their formation in biological systems, are illustrated in the figure below. Various ROS have different chemical characteristics, as permeability to cell membranes, half-life, reactivity and consequently different targets that they can react with.

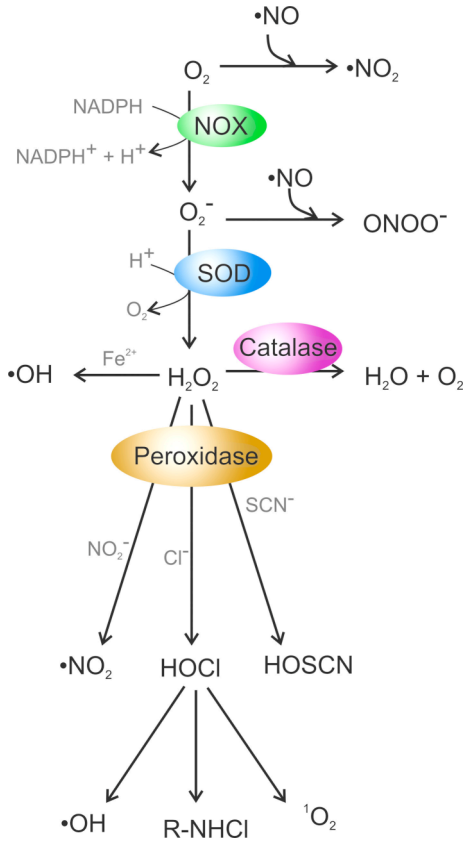

Figure 3: **The most common reactive oxygen species in vivo and their catalytic formation.** •NO: nitric oxide, $•NO_2$: nitrogen dioxide radical, $ONOO^-$: peroxynitrite, •OH, hydroxyl radical, NO_2^-: nitrite ion, HOCl: hypoclorous acid, SCN^-: thiocyanate, HOSCN: Hypothiocyanous acid, $^1O_2$: singlet oxygen, SOD: superoxide dismutase. Figure modified from (113).

In a cell ROS can be produced in several different compartments, where they achieve distinct functions. In the mitochondria ROS arise as result of leakage from the electron transport chain and have recently been found to contribute to cell signalling (reviewed in (114)). In the endoplasmic reticulum ROS are produced during protein folding, in particular in conjunction with oxidation of sulfhydryl groups to form disulfide bond, a

process mediated by a thioredoxin protein disulfide isomerase (PDI) (115). In this compartment NOX4 complex has also been described (116).
Specialized enzymatic complexes produce the majority of the ROS in the cell: the **NOX family of NADPH oxidases**, comprising of five NADPH oxidase complexes (NOX1-5), and two dual oxidase complexes (DUOX1-2) (reviewed in (117)). They mediate ROS production over the cell membranes in virtually every cell of the body, but their ROS potential, the activation mechanisms as well as their cell and tissue distribution vary greatly. Their physiological role spans from pathogen killing to signalling. A wide range of pathologies are associated with their dysfunctions, including both deficiency and increased activity (reviewed in (117)). The phagocyte NOX2 complex is the first identified and most studied complex. It has also been studied in different contexts in the investigations illustrated in this thesis. The NOX2 complex will be described in details in the next paragraphs.

THE NOX2 COMPLEX: STRUCTURE AND REGULATION

The NOX2 complex is expressed mainly in phagocytes, having the highest expression in neutrophils followed by macrophages and dendritic cells (118). Low expression has been detected in B cells and T cells (119, 120). Other cells types have also been found to express it, as neurons and cardiomyocytes (reviewed in (117)).
The NOX2 complex is localized in membranes and catalyses the transfer of two electrons from intracellular NADPH to two extracellular or luminal oxygen molecules, generating superoxide (O_2^-) (121). In phagocytes the NOX2 complex is present in both the plasma membrane and organelles membranes, as phagosomes and granules. The NOX2 complex comprises of multiple proteins in addition to the enzymatic core. The different protein components were identified and cloned over a decade ago and extensive work has been performed to understand the regulation of the complex.

Today the following structure and activation is proposed. The activation of the NOX2 complex requires of at least five components to be recruited at the membrane and assembled together. The catalytic core is constituted by two transmembrane proteins: **GP91^{phox}** (called also NOX2) and **P22^{phox}**, which together form the cytochrome *b558* complex (122). The two proteins are constitutively associated and P22^{phox} is necessary for the stability of GP91^{phox}, since in P22^{phox}-deficient patients no GP91^{phox} protein was detectable (123). Four cytosolic proteins are required for full activation of the complex: **NCF1** (or P47^{phox}), **NCF2** (or P67^{phox}), **NCF4** (or P40^{phox}) and **GTPase Rac**. The NCF denomination will be used for these proteins throughout this thesis.

Activation of the NOX2 complex requires translocation of the four factors from the cytosol to the cytochrome *b558* complex in the membrane. The first event of the activation is the phosphorylation of NCF1 and the consequent release of its auto-inhibitory conformation (125). NCF1 can then bind to P22^{phox} and translocate to the membrane (126). As NCF1 is bound to NCF2, also NCF2 get translocated to the membrane (127). NCF1 activation leads to the translocation of the other cytosolic factors, therefore it is considered as the organizer subunit. NCF2 is then able to bind GP91^{phox} (128) and activate it, therefore being considered the activator (129). The NCF4 subunit is also translocated to the membrane (130). NCF4 seems to be an important regulator of the intracellular phagocytosis-induced ROS (131, 132). Finally,

the GTPase Rac interacts directly with GP91^{phox} and with NCF2 (133, 134). Rac exists as three homologous proteins that participate in several cellular signalling mechanisms. When it comes to NOX2 complex activity, Rac1 operates in macrophages while Rac2 in neutrophils (135, 136).

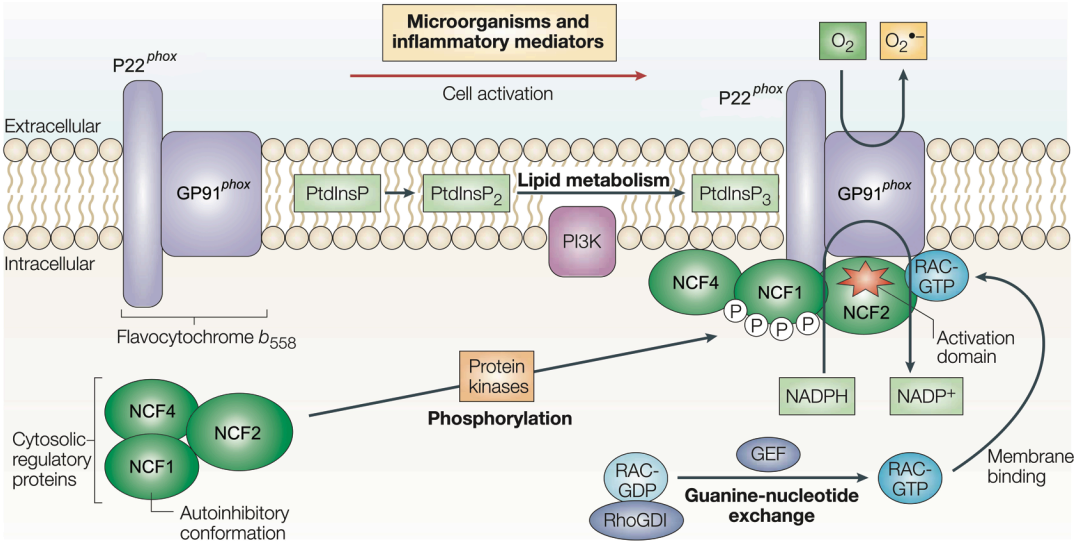

Figure 4 | **Activation of reactive oxygen species (ROS) generation by assembly of Phox regulatory proteins in phagocytes.**

Figure 4: NOX2 complex and its activation. Figure modified from (124).

In phagocytes, most of the GP91^{phox}/ P22^{phox} complex is located in intracellular compartments at resting state (137). After activation the complex can be functional in the same granule, producing intracellular ROS, or it can translocate to the plasma or the phagosome membrane as result of the fusion of the organelle were it was stored with plasma membranes (137, 138).
ROS production by the NOX2 complex is mainly regulated by the activation of the pre-existing proteins, induced transcription being only as minor way to regulate ROS production. Some signals, as cytokines and TLR agonists, can **prime** the complex and induce only the first part of the activation: the phosphorylation of NCF1 and its translocation to the membrane. This priming will accelerate and amplify the assembly of the complex once the activation signal arrives (139). Other signals, as phorbol-12-myristate-13-acetate (PMA) or formyl-methionyl-leucyl-phenylalanine (fMLF), can directly **activate** the complex (reviewed in (113)).

The human and mouse *GP91^{phox}* genes are located in the X chromosome, but the genes for the other subunits are located in different chromosomes. Their expression is mainly constitutive but can be induced by a variety signals, for example by TLR ligands, IFN-γ and PMA (140-142). Several compounds can inhibit the formation of ROS by NOX2 complex, the most common being diphenylene iodonium (DPI), but none of them has been proven specific for the complex due to interaction with other pathways and other NOX complexes (reviewed in (113)).

NCF1

NCF1 is a cytosolic, non glycosylated protein of about 47 kDa in size (143, 144). It is considered to be the organizer subunit of the NOX2 complex because the activator subunit NCF2, the small NCF4 subunit, and the GTPase Rac fail to translocate to the membrane in neutrophils lacking NCF1 (145).
NCF1 has a phox domain (PX) that interacts with membrane phospholipids, in particular to 3'-phosphorylated phosphatidylinositols, the products of phosphotidylinositol 3-kinase. It has two SH3 domains that interact with the C-terminal autoinhibitory region (AIR) in resting state but bind to proline-rich regions in the C-terminus of $P22^{phox}$ in activated state (146). After phosphorylation NCF1 undergoes a conformational change and can then bind $P22^{phox}$ and translocate to the plasma membrane (147). A proline-rich region at the C-terminus of NCF1 interacts with SH3 domains in NCF2 (129).
The *Ncf1* gene is encoded on chromosome 5 in mouse and on chromosome 7 in humans, but the gene structure differs in the two species as pseudogenes and copy number variations of *NCF1* are present in the human but not in the mouse genome (148-151). The expression is induced by cell differentiation and activation stimuli, as TLR ligands, cytokines, glutamine and PMA (140, 142, 152, 153).

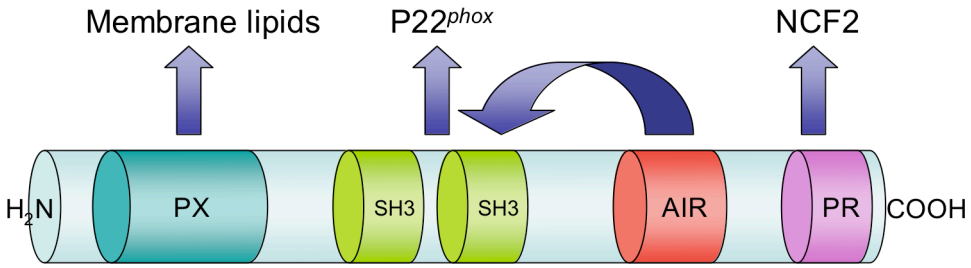

Figure 5: illustrative structure of NCF1
PX: phox domain, SH3: SRC homology 3 domain, AIR: autoinhibitory domain, PR: proline-rich domain

ANTIBACTERIAL FUNCTION OF NOX2 COMPLEX AND DISEASE IMPLICATIONS

The NOX2 complex was identified while searching for the molecular dysfunctions causing **chronic granulomatous disease (CGD)**, a condition where the patient suffers from recurrent and life-threatening infections by bacteria and fungi (154-157). Mutations in all the subunits of the complex, including NCF1, have been observed in CGD patients (158, 159). *Ncf1* knockout mice (*Ncf1-/-*) are affected by spontaneous bacterial infections (160) and therefore they are often used as a mouse model of CGD. A similar phenotype has been observed in mice carrying a natural occurring SNP in *Ncf1* (paper III). The antibacterial and antifungal activity of the complex is due to the production of ROS. The superoxide generated by the NOX2 complex can kill the pathogen by itself (117). However, the antibacterial activity of ROS is carried manly out through activation of several antimicrobial mechanisms that result in the formation of toxic compounds or activation of proteases. These mechanisms include: 1) formation of other ROS and reactive nitrogen species, either spontaneously or by enzymatic

catalysis, as suggested by the fact that CGD phagocytes can recover their killing capacity by addition of H_2O_2 generating system (161); 2) regulation of the pH: superoxide being a weak base, it increases the pH of the phagosome allowing optimal activity of the proteases in neutrophils (162); 3) activation of ion flux into the phagosome, due to an increase in negative charges inside the granule mediated by superoxide, which activates proteases (163); 4) induction of apoptosis in infected neutrophils and macrophages (164-166).

THE NOX2 COMPLEX IN INFLAMMATION AND RA

ROS is a double-edged sword: it is essential to kill pathogens, but it can also harm the tissue. In inflamed tissues, as RA joints, the NOX2 complex in neutrophils is primed, allowing them to produce more ROS and more rapidly (167-169). In these tissues increased oxidation of biological molecules and reduced levels of antioxidant system are detected (170). These observations led to the belief that high ROS production is harmful for the host.

The other side of the coin is that impairment of ROS production by the NOX2 complex is accompanied by excessive inflammation, as observed in CGD patients. The name of the disease refers to chronic persistency of granulomata, local inflammations characterized by a compact collection of cells, mainly macrophages but also other innate immune cells and lymphocytes. A part from infection-derived inflammations, CGD patients suffer from a variety of inflammatory conditions and autoimmune diseases ((171) and reviewed in (172)). Animal models for CGD, where *gp91phox* or *Ncf1* have been knocked-out or mutated, show higher susceptibility to bacterial and fungal infections ((160, 173, 174) and paper III), as well as to inflammatory and autoimmune diseases ((5, 6, 175, 176) and paper I, II and IV).

Therefore one of the functions of the NOX2 complex is to **dampen inflammation**. This is achieved through the direct activity of ROS on the resolution of the inflammation, i.e. clearing the pathogen causing the inflammation, and through the function of ROS as signalling molecules, attenuating pro-inflammatory signals and stimulating anti-inflammatory ones.

The role of NOX2 complex-derived ROS as signalling molecule has been thoroughly investigated in the last years. ROS has been found to affect a variety of cellular processes: cell growth and death, gene expression, protein activation or inactivation, ion channels, calcium signalling and angiogenesis (reviewed in (117)). So far, in immune cells, few direct targets of ROS-mediated oxidation have been identified but several inflammatory mechanisms have been found affected: they will be discussed in the following paragraphs.

For what it concerns gene expression in immune cells, a number of genes involved in the inflammatory response are regulated by ROS, including TNF-α (177), interferon (IFN)-β pathway (178), transforming growth factor (TGF)-β among others (reviewed in (179)). NOX2 complex-derived ROS affects gene expression via transcription factors: NF-κB, AP-1 and p53, which have a cysteine residue in their DNA binding site (reviewed in (117, 180)). NF-κB is a transcription factor whose activity is involved in the expression of immune response (181). NF-κB has also been implicated in pathogenesis of RA since up-regulation of NF-κB and of NF-κB regulated genes has been observed in the RA synovium (182, 183).

Central signalling pathways in immune cells that are regulated by ROS are the ones mediated by mitogen-activated protein (MAP) kinases, which are involved in anti-apoptotic signalling (184), in differentiation and activate transcription via NF-κB (reviewed in (117)).
Cell specific pathways regulated by NOX2-dependent ROS are discussed below.

Antigen presenting cells and neutrophils

NOX2 complex-dependent ROS production is central in modulating several functions of antigen presenting cells (APCs). First, ROS production is part of the physiological activation of APCs: NOX2 complex expression and activity is increased after activation stimuli, such as TLR ligands (140) and suggested to be reduced after anti-inflammatory stimuli such as IL-4 (185). The downstream effects of NOX2 complex-dependent ROS production in APCs are multiple. APCs process and present antigen to T cells to activate them. In order to produce antigens, proteases degrade proteins in a process that is dependent on the pH of the phagosome. Dendritic cells (DCs) with impaired NOX2 complex show decreased pH and increased antigen degradation, leading to defective cross-presentation to CD8+ T cells (186). This effect has not been observed during MHC class II-dependent presentation to classical CD4 T cells, the subtype of cells considered to be the main players in RA (186, 187). Macrophages derived from the *gp91phox* knock-out mouse, a model for CGD, displayed enhanced **phagosomal proteolysis** and capacity to reduce disulfide bonds compared to wt mice (188), which is in line with the observations by Savina et al. (186). No difference between *gp91phox* knock-out and wt mice was detectable when the macrophages were treated with IL-4 (185). **Cytokine production** by APCs is another signal important for T cell activation helping to shape the quality of the T cell response. Phagocytes from CGD patients released lower levels of anti-inflammatory cytokines, such as TGF-β, during phagocytosis (166). CGD leukocytes produced higher levels of pro-inflammatory cytokines, as TNF-α and IL-6, after TLR or fungal stimulation (189, 190). Also CGD neutrophils are hyperresponsive and produce higher levels of both pro-inflammatory (IL-8, TNF-α) and anti-inflammatory (IL-10) cytokines after fungal stimulation than neutrophils from healthy individuals (191).
Even B cells express the NOX2 complex, although at lower levels than phagocytes (paper I), and they produce ROS upon stimulation via Ig (192), LPS (193) and Epstein-Barr virus (194). NOX2 complex-dependent ROS production has been suggested to have an impact on B cell differentiation and activation (193).
The expression of the NOX2 complex in APCs has been reported to impact on T cell functions in autoimmune, cancer and viral infection settings, where macrophages and myeloid derived suppressor cells suppress T cell responses through NOX2 complex activity ((195, 196) and paper I and II).

T cells

Expression of the NOX2 complex and/or autonomous ROS production by T cells is still debated, as some studies have reported it while others fail to detect them (120, 197-199). TCR stimulation induced ROS production in T cells (reviewed in (200)), even when the stimuli were MHC molecules carrying the RA associated sequence called Shared epitope (201). ROS production by T cells is very minimal compared to that of

myeloid cells (202). Several T cell signalling pathways and functions are however regulated by redox balance, but their dependency on NOX2 complex-derived ROS is not clear. Among those, the most studied are several molecules downstream of the **TCR signalling pathway**, which have been associated with RA and are redox-sensitive: protein phosphatases ((203) reviewed in (204)), protein tyrosine kinases (205) and the linker for activation of T cells (LAT) (206, 207).
A protein phosphatase, encoded by the gene *PTPN22*, has been associated with several autoimmune diseases, including RA (31). Phosphatases are inhibited by oxidation due to the presence of a cysteine in their reactive site (203, 204).
T cells from the synovium are activated but hyporesponsive to TCR stimulation *in vitro*. This phenotype has been associated with a conformation change of the protein tyrosine kinase LCK that occurs in synovial fluid T cells of RA patients but not in blood T cells (208). Several kinases have been shown to be activated by ROS but it is unclear the role of NOX2 complex-dependent ROS (reviewed in (117)).
Similarly, hyporesponsiveness to TCR stimulation of RA synovium T cells has been linked to oxidation of LAT, which induces its translocation to the cytosol and therefore inactivation (206, 207).
Recently a direct influence of NOX2 and NCF1 on T cell phenotype has been reported. Defects in the NOX2 complex enhance production of pro-inflammatory **cytokines** in human CGD leukocytes (189). Also in mouse models of CGD, T cells exhibit increased production of IL-17 and IL-23 but decreased IFN-y, IL-10 and TGF-β (209). This response has been described as a consequence of a dysfunctional kynurenine pathway in the tryptophan catabolism, which suppresses T cells activation (209). However, the same pathway is not found affected in human CGD leukocytes (210). In another mouse strain lacking NOX2 complex-derived ROS due to a natural occurring SNP in *Ncf1*, T cells produced higher amounts of IFN-γ, TNF-α (paper I), IL-17 (211) and IL-5 (212). In rat, a SNP in *Ncf1* that decreases ROS production increases numbers of reduced protein on the surface of T cells and the arthritogenicity of the cells (197). Recently it has been shown how defects in *Ncf1* impair development and functionality of T regulatory cells (198, 209).
In both human and rodents, abundant evidence is starting to reveal the regulatory molecular mechanisms of NOX2 complex and derived ROS on T cells activity, pointing towards a role of ROS in suppression of T cell activation.

NCF1 in RA

Ncf1 was positionally cloned in 2003 as an arthritis-regulating gene in the pristane–induced rat model of arthritis, a model of autoimmune arthritis (5). DA rats showed **lower ROS production** and **higher severity** of the disease compared to the E3 strain (5). Out of the three SNPs that differ between the DA and E3 form of *Ncf1*, one has been recently identified as the responsible SNP for causing both the reduced ROS production and more severe arthritis (213). This SNP does neither affect localization of the protein nor the assembly of the NCF1/NCF2 complex (213), but is likely to operate later in the activation cascade of the NOX2 complex. In mice, *Ncf1* knock-out animals developed a more severe arthritis when injected with intra-articular with zymosan (176). A spontaneously occurred mutation that impairs the function of the NCF1 protein increases arthritis severity, antibody production and pro-inflammatory cytokines production in CIA (6, 212, 214), and in peptide-induced arthritis (paper IV).

This *Ncf1* mutation (*Ncf1*^{m1J} or *Ncf1*/*)* facilitates the breaking of tolerance to collagen and development of autoimmune arthritis ((215-217) and paper II).
Recently a copy number variation of the *NCF1* gene has been found to be associated with RA (151). NCF1 is found phosphorylated in neutrophils from synovial fluid of RA patients, while it is not in circulating blood neutrophils. Synovial fluid neutrophils produce more ROS and high local levels of TNF-α and IL-8 (169). This observation suggests that the NOX2 complex is primed in synovium of RA patients, and it agrees with the observation of a chronic NOX2 complex activation in the joints of RA patients (167, 168).
All these observation strongly suggest that the function of NOX2 complex and the regulation by NCF1 are important in RA pathogenesis. In animal models, impairment of NCF1, and therefore of NOX2 complex-dependent ROS production, increases severity and susceptibility to arthritis. This is accompanied by an increased T cells activation. Our current hypothesis is that APCs-derived ROS shape the T cell response, suppressing a pathogenic autoimmune activity. Paper I investigated this hypothesis and confirmed it.
In humans, recent investigations confirmed the correlation between higher severity of autoimmune inflammation in the nervous system and lower NOX2 complex-dependent ROS production by leukocytes (218-220). Similar studies performed with RA patients are attending.
Identifying the molecular mechanisms behind the influence of NCF1 in arthritis will lead to a deeper understanding of the disease and the development of targeted drugs.

PRESENT INVESTIGATION

Ncf1 was found to be an arthritis-regulating gene in rats and mice: natural occurring SNPs in the gene reduced the functionality of the protein and increased the severity of arthritis in rat and mouse models (5, 6). An effect of the *Ncf1* mutation was the increased activation of autoreactive and arthritogenic T cells (5, 6, 197). As neither NCF1 expression nor NCF1-dependent oxidative burst was detected in T cells (197, 199, 202), we hypothesized that ROS produced by other cells would influence the T cell phenotype. T cells interact closely with APCs throughout all their life. APCs - a classification that includes macrophages, dendritic cells and B cells - express NCF1 and are able to produce an NCF1-dependent oxidative burst (140, 199, 221, 222). After observing that macrophages were the cells that expressed the highest levels of NCF1 and produced the most ROS among APCs, we investigated how NCF1-dependent ROS by macrophages shaped the adaptive immune response. We found that ROS production by macrophages reduced the autoimmune response by decreasing the activation of Th1 T cells (paper I). We then confirmed that macrophages lacking NCF1-dependent ROS production could present antigens and prime an autoimmune response (paper II). NCF1 is a regulatory component of the NOX2 complex, whose function is vital for anti-bacterial defence. Neutrophils, the cells with the highest expression of NOX2 complex, have been considered the main players in the fight against bacterial infections. We could show that NCF1-dependent ROS production by macrophages alone could alone efficiently protect the host from bacterial infections (paper III). In the last paper, we investigated a new model of arthritis where the disease could be induced by a peptide instead of a protein as in CIA. Even in this model, the disease severity was increased by the *Ncf1* mutation. We characterized the immunological pathways involved in the pathogenesis of the arthritis and the features that render the peptide arthritogenic (paper IV).

PAPER I

In paper I we first confirmed that in C57BL/10.Q (B10.Q) mice, the used background strain, all APCs expressed NCF1 and were able to produce ROS upon stimulation. The level of protein expression and of ROS production was different in different cell types, where macrophages were the cells with the highest expression followed by dendritic cells and B cells. We chose then to investigate the effect of macrophages oxidative burst *in vivo*. We constructed a transgenic mouse where the functional NCF1 protein was expressed under the human CD68 promoter on *Ncf1* mutated (*Ncf1*/**) background. The new strain was named macrophage NCF1 (MN). The expression of the MN transgene on *Ncf1*/** background restored NCF1 expression and ROS production specifically in macrophages. *Ncf1*/* MN+* mice immunized with CII in CFA developed less severe arthritis and lower titers of anti-CII antibodies than their littermates *Ncf1*/* MN-* and at comparable levels to *Ncf1*/+* mice. No differences in arthritis score and incidence were observed between *Ncf1*/* MN+* and *Ncf1*/* MN-* mice when CAIA, a T cell-independent model of arthritis, was induced. T cells from *Ncf1*/* MN+* mice produced lower levels of IL-2 and Th1 cytokines after *in vitro* re-challenge, when compared to cells from *Ncf1*/* MN-* mice. In a criss-cross experiment where primed T cells and macrophages from *Ncf1*/* MN+*, *Ncf1*/* MN-* or *Ncf1+/+*

(*Ncf1 wt*) mice were co-cultured *in vitro* in all possible combinations, ROS producing macrophages decreased the proliferation and IL-2 production of T cells, independently of the T cell origin. Conversely, IFN-γ production was decreased in all co-cultures where T cells originated from the *Ncf1**/* *MN*+ or *Ncf1*+/+ mice, independently of macrophages origin.
From this study we could conclude that ROS production by macrophages decreases T cell activation and Th1 polarization, leading to a limited T cell dependent autoimmune outbreak. We could identify a role for macrophage-derived ROS in both education and activation of T cells. Macrophage-derived ROS dampens proliferation during re-challenge, irrespective of the T cells origin, suggesting a short lasting effect of ROS on T cells activation. On the contrary, macrophage-derived ROS during selection and priming decreases the Th1 response in T cells, indicating a long lasting effect of ROS during T cell education.

PAPER II

In paper II we aimed at investigating whether macrophages could prime arthritogenic T cells. We took advantage of the well-characterized CIA model, where the MHC class II A^q molecule is associated with susceptibility to disease and A^p with resistance (76, 78). This is most likely due to the fact that APCs expressing A^q can process and present the immunogen, CII, to T cells while APCs carrying A^p cannot (77). At molecular level, A^q binds with higher affinity to the immunodominant T cell epitope than A^p (77). The *Ncf1* mutation can increase the arthritis severity in A^q expressing mice (B10.Q) but could not break the resistance to arthritis of A^p expressing C57BL/10.P mice (B10.P), suggesting that the pathways altered by *Ncf1* mutation could not break the arthritis resistance imposed by the MHC. In order to study CII presentation by macrophages we constructed a transgenic mouse where the A^q molecule is expressed under the human CD68 promoter, considered being specific for macrophages in mouse (223), on the B10.P background (Macrophage $A\beta^q$: MBQ). We confirmed that in MBQ mice macrophages were the only APCs that expressed A^q and could present CII to T cells. In order to study the role of *Ncf1* during macrophage priming, the *Ncf1* mutation was inserted. When CIA was induced in MBQ mice, with or without the *Ncf1* mutation, only the mice carrying the mutated form of *Ncf1* developed disease. We therefore concluded that macrophages could present CII to T cells and prime them to be arthritogenic. This occurs only when NCF1-dependent ROS is impaired, indicating that ROS regulates the T cells response at the selection and/or priming stage.

PAPER III

In paper III we investigated the influence of macrophage specific expression of NCF1 on the best-known function of the NOX2 complex: antimicrobial defence. The NADPH complex was discovered in the ’70s when trying to understand the molecular basis of a disease called chronic granulomatous disease (CGD) (154), characterized by recurred life threatening infection and a reduced oxidative burst in phagocytes (155-157). Mutations in all the subunits of the complex, including NCF1, have been observed in CGD patients (158, 159). *Ncf1* knockout mice (*Ncf1-/-*) are affected by spontaneous bacterial infections (160) and therefore are often used as a mouse model of CGD. As human CGD is most commonly caused by single mutations, we investigated whether

the naturally occurring *Ncf1* mutated (*Ncf1**/*) mouse showed increased susceptibility to infections comparably to the human CGD condition. We observed that *Ncf1**/* mice occasionally developed spontaneous infections in soft tissues and that they were not able to resolve the inflammation. We isolated the bacteria from infected areas and identified them as *Staphylococcus xylosus*, the same strain found in *Ncf1*-/- mice, and *S. aureus*, the most common CGD pathogen. We then confirmed that *Ncf1**/* mice were more susceptible to these bacterial infections than *Ncf1*+/+ mice when the bacteria were intravenously administered. As neutrophils are the cells that express the highest amount of NOX2 complex (and NCF1 protein) and they produce the most prominent oxidative burst, they have been extensively studied for their antimicrobial activity and considered the main players in the ROS mediated defence against pathogens (2). Monocytes and macrophages have an important capacity to phagocytose and kill pathogens. They also express the NOX2 complex and produce oxidative burst. We wanted to know if monocytes and macrophages alone could control bacterial infections via ROS production. Since in both CGD patients and mouse models the NOX2 complex is deficient in all cells, it was not possible to delineate the specific contribution of macrophage NOX2 to host defence *in vivo*. We took advantage of the MN mouse, where functional *Ncf1* is encoded under the human CD68 promoter on *Ncf1**/* background and therefore specifically expressed in monocytes and macrophages, but not in neutrophils, to dissect the role of monocytes in antibacterial defence. We could show that *Ncf1**/* *MN*+ mice were resistant to induced infections with *S. xylosus* and *Burkholderia cepacia*, a common pathogen in CGD patients, which were lethal for *Ncf1**/* *MN*- mice.

In this paper we concluded that the naturally occurring mutation in *Ncf1* predisposes to a CGD-like disease in mice, characterized by the inability to resolve bacterial infection and the development of chronic inflammation, ultimately leading to death. We could also conclude that monocyte/macrophage restricted expression of NCF1 protected mice from lethal infection, therefore highlighting the role of this cell type in the innate immunity function of antibacterial defence.

PAPER IV

In paper IV we investigated a new model of arthritis where the disease is induced with a peptide instead of a protein in *Ncf1**/* and *Ncf1*+/+ B10.Q mice. The ultimate goal is to understand how NCF1-dependent ROS affects antigen processing and presentation. In this paper we characterized the immunological pathways crucial for disease development. The peptide used is derived from the glucose-6-phosphate isomerase protein (GPI). It was identified as arthritogenic in DBA/1 mice in a screening for GPI peptides that would theoretically bind the MHC II H-2^q (the same in DBA/1 mice and B10.Q mice) (111). The GPI protein was found to be a target of the immune response in the spontaneous model of arthritis K/BxN mice, a TCR transgenic mouse where T cells recognise peptides derived from the murine GPI protein (96). Immunization with human GPI protein leads to severe arthritis on DBA/1 background, but not on B10.Q (105, 106). The arthritogenic peptide isolated from the human GPI protein spans from amino acid 325 to 339 ($hGPI_{325-339}$) and has as sequence: H-IWYINCFGCETHAML-OH (111).

We first wondered whether the $hGPI_{325-339}$ peptide could induce arthritis in B10.Q mice and whether the severity of the disease was affected by the *Ncf1* mutation. We observed

that $hGPI_{325-339}$ peptide induced arthritis in B10.Q mice and the severity of the disease was increased by the *Ncf1* mutation. We continued by assessing the immunological pathways that underlie the pathology of the disease in B10.Q.*Ncf1**/* mice. We could observe that the $hGPI_{325-339}$ peptide-induced arthritis was dependent on MHC haplotype (with increasing severity according to the haplotype: q>p>r) and on B and T cells, but not on C5. Immunization with the peptide induced a specific Th1 and Th17 response with antibody production against the $hGPI_{325-339}$ peptide. T cells and B cells cross-reacted to the murine (m)$GPI_{325-339}$ peptide and murine protein. The $hGPI_{325-339}$ peptide differs from $mGPI_{325-339}$ peptide in two amino acids at positions 331 and 338, underlined in the sequence ($mGPI_{325-339}$: H-IWYINCYGCETHALL-OH). The murine peptide was not able to induce arthritis. We could confirm that the amino acid in position 331 was the one responsible for the arthritogenicity of the $hGPI_{325-339}$ peptide. At molecular level this could be explained by a higher affinity to MHC of the peptide containing phenylalanine (F) compared to the one containing tyrosine (Y). *Ncf1**/* mutated mice developed a very mild disease after immunization with $mGPI_{325-339}$ peptide and could prime anti-$mGPI_{325-339}$ peptide Th1 and Th17 cells, confirming that the mutation in *Ncf1* decreases tolerance towards self antigens.

From this study we concluded that a peptide-induced arthritis could be established in B10.Q and B10.Q.*Ncf1**/* mice. This disease model shares important features with protein-induced arthritis models, as CIA, indicating common pathways in all the models. We could confirm a MHC dependency of the disease and that the peptide binds MHC II H-2^q, as postulated by Iwanami et al, and as it is occurs with CII. The disease was dependent on the presence of an intact adaptive immune compartment, as CIA and GPI protein-induced arthritis. The immunization with $hGPI_{325-339}$ in CFA induced activation of Th1 and Th17 cells and B cells producing anti-$hGPI_{325-339}$ IgG, as in the other models. T and B cells cross-reacted with the mGPI protein, suggesting this protein as a candidate target for autoimmunity *in vivo*.

We could dissect the peptide sequence and link the arthritogenicity of the peptide on the presence of phenylalanine in position 331 which also increased the binding to MHC A^q.

Finally we could confirm that a mutation in *Ncf1* decreased the tolerance level also in peptide-induced arthritis, predisposing mice to autoimmunity as observed earlier in other models of arthritis (212, 216, 217).

CONCLUDING REMARKS AND FUTURE PROSPECTIVE

Finding the mechanisms that explain how a disease-associated gene mediates its effect is the main challenge of today's medical research. Nowadays technical advances allow to identify a large number of genes that have a small impact on disease and the task has moved from investigating which genes are associated with the disease to how these genes operate. Mechanistic studies have the goal to describe "who, where, when, what and how" the associated gene influences the disease development. Many of these studies can be performed *in vitro*, using cell lines or tissue extracts. But to get a complete 4D picture of the pathologic mechanism animal models of disease are a unique and still indispensable tool. They give the possibility to test the function of the gene in an *in vivo* setting that resembles the human situation, even though is not identical. Animal models allow to study specifically and to modify all the parameters ("who, where, when, what and how") that are believed to be of importance, most of which cannot be altered in the human situation. In this study, animal models allowed to assess the relevance of a cell type, macrophages, and of a biochemical process, the production of NOX2 complex-dependent ROS, at the same time in several disease settings. As a result, we could confirm that macrophages play an essential role in the immune system, both in the innate and adaptive responses. They mediate both known functions of the NOX2 complex-derived ROS: killing of bacteria and down-regulation of T cell mediated inflammation.
We could also validate a new disease model, a peptide-induced arthritis, that shares important characteristics with the human disease and previous models, while being characterized by a higher degree of simplicity compared to previous models.

These discoveries set a new starting point and give new tools to investigate the molecular pathways underlying arthritis. Since macrophage-derived ROS play an important role in inflammation, it is possible to use this transgenic system to study the mechanisms regulated by ROS in T cells and/or macrophages. The peptide-induced arthritis model will allow a close examination of the features that render a peptide arthritogenic, and of the role of ROS in the development of inflammatory response towards an antigen. The ultimate goal and hope is to identify pathways and processes that can be modified or reverted so to stop the disease progression.

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REFERENCES

1. Himmelfarb, J., Stenvinkel, P., Ikizler, T.A., and Hakim, R.M. The elephant in uremia: oxidant stress as a unifying concept of cardiovascular disease in uremia. *Kidney Int*. 2002; 62:1524-1538.
2. Quie, P.G., White, J.G., Holmes, B., and Good, R.A. In vitro bactericidal capacity of human polymorphonuclear leukocytes: diminished activity in chronic granulomatous disease of childhood. *J Clin Invest*. 1967; 46:668-679.
3. Cooper, G.S., Bynum, M.L., and Somers, E.C. Recent insights in the epidemiology of autoimmune diseases: improved prevalence estimates and understanding of clustering of diseases. *J Autoimmun*. 2009; 33:197-207.
4. Gabriel, S.E., and Michaud, K. Epidemiological studies in incidence, prevalence, mortality, and comorbidity of the rheumatic diseases. *Arthritis Res Ther*. 2009; 11:229.
5. Olofsson, P., Holmberg, J., Tordsson, J., Lu, S., Åkerström, B., and Holmdahl, R. Positional identification of Ncf1 as a gene that regulates arthritis severity in rats. *Nat Genet*. 2003; 33:25-32.
6. Hultqvist, M., Olofsson, P., Holmberg, J., Bäckström, B.T., Tordsson, J., and Holmdahl, R. Enhanced autoimmunity, arthritis, and encephalomyelitis in mice with a reduced oxidative burst due to a mutation in the Ncf1 gene. *Proc Natl Acad Sci U S A*. 2004; 101:12646-12651.
7. Pincus, T., Callahan, L.F., Sale, W.G., Brooks, A.L., Payne, L.E., and Vaughn, W.K. Severe functional declines, work disability, and increased mortality in seventy-five rheumatoid arthritis patients studied over nine years. *Arthritis Rheum*. 1984; 27:864-872.
8. Londei, M., Savill, C.M., Verhoef, A., Brennan, F., Leech, Z.A., Duance, V., Maini, R.N., and Feldmann, M. Persistence of collagen type II-specific T-cell clones in the synovial membrane of a patient with rheumatoid arthritis. *Proc Natl Acad Sci U S A*. 1989; 86:636-640.
9. Snir, O., Rieck, M., Gebe, J.A., Yue, B.B., Rawlings, C.A., Nepom, G., Malmstrom, V., and Buckner, J.H. Identification and functional characterization of T cells reactive to citrullinated vimentin in HLA-DRB1*0401-positive humanized mice and rheumatoid arthritis patients. *Arthritis Rheum*. 2011; 63:2873-2883.
10. Andriopoulos, N.A., Mestecky, J., Miller, E.J., and Bennett, J.C. Antibodies to human native and denatured collagens in synovial fluids of patients with rheumatoid arthritis. *Clin Immunol Immunopathol*. 1976; 6:209-212.
11. Andriopoulos, N.A., Mestecky, J., Miller, E.J., and Bradley, E.L. Antibodies to native and denatured collagens in sera of patients with rheumatoid arthritis. *Arthritis Rheum*. 1976; 19:613-617.
12. van Boekel, M.A., Vossenaar, E.R., van den Hoogen, F.H., and van Venrooij, W.J. Autoantibody systems in rheumatoid arthritis: specificity, sensitivity and diagnostic value. *Arthritis Res*. 2002; 4:87-93.
13. Aho, K., Heliovaara, M., Maatela, J., Tuomi, T., and Palosuo, T. Rheumatoid factors antedating clinical rheumatoid arthritis. *J Rheumatol*. 1991; 18:1282-1284.
14. Waaler, E. On the occurrence of a factor in human serum activating the specific agglutintion of sheep blood corpuscles. 1939. *APMIS*. 2007; 115:422-438; discussion 439.
15. Rantapaa-Dahlqvist, S., de Jong, B.A., Berglin, E., Hallmans, G., Wadell, G., Stenlund, H., Sundin, U., and van Venrooij, W.J. Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis. *Arthritis Rheum*. 2003; 48:2741-2749.
16. Arnett, F.C., Edworthy, S.M., Bloch, D.A., McShane, D.J., Fries, J.F., Cooper, N.S., Healey, L.A., Kaplan, S.R., Liang, M.H., Luthra, H.S*., et al.* The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum*. 1988; 31:315-324.
17. Aletaha, D., Neogi, T., Silman, A.J., Funovits, J., Felson, D.T., Bingham, C.O., 3rd, Birnbaum, N.S., Burmester, G.R., Bykerk, V.P., Cohen, M.D*., et al.* 2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European

League Against Rheumatism collaborative initiative. *Arthritis Rheum*. 2010; 62:2569-2581.
18. Firestein, G.S., Alvaro-Gracia, J.M., and Maki, R. Quantitative analysis of cytokine gene expression in rheumatoid arthritis. *J Immunol*. 1990; 144:3347-3353.
19. Neumann, E., Barnum, S.R., Tarner, I.H., Echols, J., Fleck, M., Judex, M., Kullmann, F., Mountz, J.D., Scholmerich, J., Gay, S.*, et al.* Local production of complement proteins in rheumatoid arthritis synovium. *Arthritis Rheum*. 2002; 46:934-945.
20. Janossy, G., Panayi, G., Duke, O., Bofill, M., Poulter, L.W., and Goldstein, G. Rheumatoid arthritis: a disease of T-lymphocyte/macrophage immunoregulation. *Lancet*. 1981; 2:839-842.
21. Duke, O., Panayi, G.S., Janossy, G., and Poulter, L.W. An immunohistological analysis of lymphocyte subpopulations and their microenvironment in the synovial membranes of patients with rheumatoid arthritis using monoclonal antibodies. *Clin Exp Immunol*. 1982; 49:22-30.
22. Young, C.L., Adamson, T.C., 3rd, Vaughan, J.H., and Fox, R.I. Immunohistologic characterization of synovial membrane lymphocytes in rheumatoid arthritis. *Arthritis Rheum*. 1984; 27:32-39.
23. Tarkowski, A., Klareskog, L., Carlsten, H., Herberts, P., and Koopman, W.J. Secretion of antibodies to types I and II collagen by synovial tissue cells in patients with rheumatoid arthritis. *Arthritis Rheum*. 1989; 32:1087-1092.
24. Griffiths, H.R. Is the generation of neo-antigenic determinants by free radicals central to the development of autoimmune rheumatoid disease? *Autoimmun Rev*. 2008; 7:544-549.
25. MacGregor, A.J., Snieder, H., Rigby, A.S., Koskenvuo, M., Kaprio, J., Aho, K., and Silman, A.J. Characterizing the quantitative genetic contribution to rheumatoid arthritis using data from twins. *Arthritis Rheum*. 2000; 43:30-37.
26. van der Woude, D., Houwing-Duistermaat, J.J., Toes, R.E., Huizinga, T.W., Thomson, W., Worthington, J., van der Helm-van Mil, A.H., and de Vries, R.R. Quantitative heritability of anti-citrullinated protein antibody-positive and anti-citrullinated protein antibody-negative rheumatoid arthritis. *Arthritis Rheum*. 2009; 60:916-923.
27. Stastny, P. Mixed lymphocyte cultures in rheumatoid arthritis. *J Clin Invest*. 1976; 57:1148-1157.
28. Gregersen, P.K., Silver, J., and Winchester, R.J. The shared epitope hypothesis. An approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis. *Arthritis Rheum*. 1987; 30:1205-1213.
29. Deighton, C.M., Walker, D.J., Griffiths, I.D., and Roberts, D.F. The contribution of HLA to rheumatoid arthritis. *Clin Genet*. 1989; 36:178-182.
30. Suzuki, A., Yamada, R., Chang, X., Tokuhiro, S., Sawada, T., Suzuki, M., Nagasaki, M., Nakayama-Hamada, M., Kawaida, R., Ono, M.*, et al.* Functional haplotypes of PADI4, encoding citrullinating enzyme peptidylarginine deiminase 4, are associated with rheumatoid arthritis. *Nat Genet*. 2003; 34:395-402.
31. Begovich, A.B., Carlton, V.E., Honigberg, L.A., Schrodi, S.J., Chokkalingam, A.P., Alexander, H.C., Ardlie, K.G., Huang, Q., Smith, A.M., Spoerke, J.M.*, et al.* A missense single-nucleotide polymorphism in a gene encoding a protein tyrosine phosphatase (PTPN22) is associated with rheumatoid arthritis. *Am J Hum Genet*. 2004; 75:330-337.
32. Vang, T., Congia, M., Macis, M.D., Musumeci, L., Orru, V., Zavattari, P., Nika, K., Tautz, L., Tasken, K., Cucca, F.*, et al.* Autoimmune-associated lymphoid tyrosine phosphatase is a gain-of-function variant. *Nat Genet*. 2005; 37:1317-1319.
33. Rieck, M., Arechiga, A., Onengut-Gumuscu, S., Greenbaum, C., Concannon, P., and Buckner, J.H. Genetic variation in PTPN22 corresponds to altered function of T and B lymphocytes. *J Immunol*. 2007; 179:4704-4710.
34. Plenge, R.M., Padyukov, L., Remmers, E.F., Purcell, S., Lee, A.T., Karlson, E.W., Wolfe, F., Kastner, D.L., Alfredsson, L., Altshuler, D.*, et al.* Replication of putative candidate-gene associations with rheumatoid arthritis in >4,000 samples from North America and Sweden: association of susceptibility with PTPN22, CTLA4, and PADI4. *Am J Hum Genet*. 2005; 77:1044-1060.
35. Kurreeman, F.A., Padyukov, L., Marques, R.B., Schrodi, S.J., Seddighzadeh, M., Stoeken-Rijsbergen, G., van der Helm-van Mil, A.H., Allaart, C.F., Verduyn, W.,

Houwing-Duistermaat, J., *et al.* A candidate gene approach identifies the TRAF1/C5 region as a risk factor for rheumatoid arthritis. *PLoS Med*. 2007; 4:e278.
36. Remmers, E.F., Plenge, R.M., Lee, A.T., Graham, R.R., Hom, G., Behrens, T.W., de Bakker, P.I., Le, J.M., Lee, H.S., Batliwalla, F., *et al.* STAT4 and the risk of rheumatoid arthritis and systemic lupus erythematosus. *N Engl J Med*. 2007; 357:977-986.
37. Raychaudhuri, S., Remmers, E.F., Lee, A.T., Hackett, R., Guiducci, C., Burtt, N.P., Gianniny, L., Korman, B.D., Padyukov, L., Kurreeman, F.A., *et al.* Common variants at CD40 and other loci confer risk of rheumatoid arthritis. *Nat Genet*. 2008; 40:1216-1223.
38. Vessey, M.P., Villard-Mackintosh, L., and Yeates, D. Oral contraceptives, cigarette smoking and other factors in relation to arthritis. *Contraception*. 1987; 35:457-464.
39. Linn-Rasker, S.P., van der Helm-van Mil, A.H., van Gaalen, F.A., Kloppenburg, M., de Vries, R.R., le Cessie, S., Breedveld, F.C., Toes, R.E., and Huizinga, T.W. Smoking is a risk factor for anti-CCP antibodies only in rheumatoid arthritis patients who carry HLA-DRB1 shared epitope alleles. *Ann Rheum Dis*. 2006; 65:366-371.
40. Klareskog, L., Stolt, P., Lundberg, K., Kallberg, H., Bengtsson, C., Grunewald, J., Ronnelid, J., Harris, H.E., Ulfgren, A.K., Rantapaa-Dahlqvist, S., *et al.* A new model for an etiology of rheumatoid arthritis: smoking may trigger HLA-DR (shared epitope)-restricted immune reactions to autoantigens modified by citrullination. *Arthritis Rheum*. 2006; 54:38-46.
41. Hitchon, C.A., Chandad, F., Ferucci, E.D., Willemze, A., Ioan-Facsinay, A., van der Woude, D., Markland, J., Robinson, D., Elias, B., Newkirk, M., *et al.* Antibodies to porphyromonas gingivalis are associated with anticitrullinated protein antibodies in patients with rheumatoid arthritis and their relatives. *J Rheumatol*37:1105-1112.
42. de Pablo, P., Dietrich, T., and McAlindon, T.E. Association of periodontal disease and tooth loss with rheumatoid arthritis in the US population. *J Rheumatol*. 2008; 35:70-76.
43. Wegner, N., Wait, R., Sroka, A., Eick, S., Nguyen, K.A., Lundberg, K., Kinloch, A., Culshaw, S., Potempa, J., and Venables, P.J. Peptidylarginine deiminase from Porphyromonas gingivalis citrullinates human fibrinogen and alpha-enolase: implications for autoimmunity in rheumatoid arthritis. *Arthritis Rheum*. 2010; 62:2662-2672.
44. Rosenstein, E.D., Weissmann, G., and Greenwald, R.A. Porphyromonas gingivalis, periodontitis and rheumatoid arthritis. *Med Hypotheses*. 2009; 73:457-458.
45. Liao, F., Li, Z., Wang, Y., Shi, B., Gong, Z., and Cheng, X. Porphyromonas gingivalis may play an important role in the pathogenesis of periodontitis-associated rheumatoid arthritis. *Med Hypotheses*. 2009; 72:732-735.
46. Farr, S.L., Folger, S.G., Paulen, M.E., and Curtis, K.M. Safety of contraceptive methods for women with rheumatoid arthritis: a systematic review. *Contraception*. 2010; 82:64-71.
47. Pikwer, M., Bergstrom, U., Nilsson, J.A., Jacobsson, L., Berglund, G., and Turesson, C. Breast feeding, but not use of oral contraceptives, is associated with a reduced risk of rheumatoid arthritis. *Ann Rheum Dis*. 2009; 68:526-530.
48. Karlson, E.W., Mandl, L.A., Hankinson, S.E., and Grodstein, F. Do breast-feeding and other reproductive factors influence future risk of rheumatoid arthritis? Results from the Nurses' Health Study. *Arthritis Rheum*. 2004; 50:3458-3467.
49. Jorgensen, C., Picot, M.C., Bologna, C., and Sany, J. Oral contraception, parity, breast feeding, and severity of rheumatoid arthritis. *Ann Rheum Dis*. 1996; 55:94-98.
50. Kallberg, H., Jacobsen, S., Bengtsson, C., Pedersen, M., Padyukov, L., Garred, P., Frisch, M., Karlson, E.W., Klareskog, L., and Alfredsson, L. Alcohol consumption is associated with decreased risk of rheumatoid arthritis: results from two Scandinavian case-control studies. *Ann Rheum Dis*. 2009; 68:222-227.
51. Benito-Garcia, E., Feskanich, D., Hu, F.B., Mandl, L.A., and Karlson, E.W. Protein, iron, and meat consumption and risk for rheumatoid arthritis: a prospective cohort study. *Arthritis Res Ther*. 2007; 9:R16.
52. Pattison, D.J., and Winyard, P.G. Dietary antioxidants in inflammatory arthritis: do they have any role in etiology or therapy? *Nat Clin Pract Rheumatol*. 2008; 4:590-596.
53. Bengtsson, C., Nordmark, B., Klareskog, L., Lundberg, I., and Alfredsson, L. Socioeconomic status and the risk of developing rheumatoid arthritis: results from the Swedish EIRA study. *Ann Rheum Dis*. 2005; 64:1588-1594.

54. Pedersen, M., Jacobsen, S., Klarlund, M., and Frisch, M. Socioeconomic status and risk of rheumatoid arthritis: a Danish case-control study. *J Rheumatol*. 2006; 33:1069-1074.
55. Hurkmans, E., van der Giesen, F.J., Vliet Vlieland, T.P., Schoones, J., and Van den Ende, E.C. Dynamic exercise programs (aerobic capacity and/or muscle strength training) in patients with rheumatoid arthritis. *Cochrane Database Syst Rev*. 2009:CD006853.
56. Chalmers, P.N., Sherman, S.L., Raphael, B.S., and Su, E.P. Rheumatoid synovectomy: does the surgical approach matter? *Clin Orthop Relat Res*. 2011; 469:2062-2071.
57. Kirwan, J.R., Bijlsma, J.W., Boers, M., and Shea, B.J. Effects of glucocorticoids on radiological progression in rheumatoid arthritis. *Cochrane Database Syst Rev*. 2007:CD006356.
58. Blake, D.R., Robson, P., Ho, M., Jubb, R.W., and McCabe, C.S. Preliminary assessment of the efficacy, tolerability and safety of a cannabis-based medicine (Sativex) in the treatment of pain caused by rheumatoid arthritis. *Rheumatology (Oxford)*. 2006; 45:50-52.
59. Johnston, A., Gudjonsson, J.E., Sigmundsdottir, H., Ludviksson, B.R., and Valdimarsson, H. The anti-inflammatory action of methotrexate is not mediated by lymphocyte apoptosis, but by the suppression of activation and adhesion molecules. *Clin Immunol*. 2005; 114:154-163.
60. Phillips, D.C., Woollard, K.J., and Griffiths, H.R. The anti-inflammatory actions of methotrexate are critically dependent upon the production of reactive oxygen species. *Br J Pharmacol*. 2003; 138:501-511.
61. Phillips, D.C., Dias, H.K., Kitas, G.D., and Griffiths, H.R. Aberrant reactive oxygen and nitrogen species generation in rheumatoid arthritis (RA): causes and consequences for immune function, cell survival, and therapeutic intervention. *Antioxid Redox Signal*12:743-785.
62. Hyrich, K.L., Symmons, D.P., Watson, K.D., and Silman, A.J. Comparison of the response to infliximab or etanercept monotherapy with the response to cotherapy with methotrexate or another disease-modifying antirheumatic drug in patients with rheumatoid arthritis: results from the British Society for Rheumatology Biologics Register. *Arthritis Rheum*. 2006; 54:1786-1794.
63. Nishimoto, N., Yoshizaki, K., Miyasaka, N., Yamamoto, K., Kawai, S., Takeuchi, T., Hashimoto, J., Azuma, J., and Kishimoto, T. Treatment of rheumatoid arthritis with humanized anti-interleukin-6 receptor antibody: a multicenter, double-blind, placebo-controlled trial. *Arthritis Rheum*. 2004; 50:1761-1769.
64. Cohen, S.B. The use of anakinra, an interleukin-1 receptor antagonist, in the treatment of rheumatoid arthritis. *Rheum Dis Clin North Am*. 2004; 30:365-380, vii.
65. McInnes, I.B., and Gracie, J.A. Interleukin-15: a new cytokine target for the treatment of inflammatory diseases. *Curr Opin Pharmacol*. 2004; 4:392-397.
66. Reff, M.E., Carner, K., Chambers, K.S., Chinn, P.C., Leonard, J.E., Raab, R., Newman, R.A., Hanna, N., and Anderson, D.R. Depletion of B cells in vivo by a chimeric mouse human monoclonal antibody to CD20. *Blood*. 1994; 83:435-445.
67. Kremer, J.M., Westhovens, R., Leon, M., Di Giorgio, E., Alten, R., Steinfeld, S., Russell, A., Dougados, M., Emery, P., Nuamah, I.F*., et al.* Treatment of rheumatoid arthritis by selective inhibition of T-cell activation with fusion protein CTLA4Ig. *N Engl J Med*. 2003; 349:1907-1915.
68. Singh, J.A., Christensen, R., Wells, G.A., Suarez-Almazor, M.E., Buchbinder, R., Lopez-Olivo, M.A., Ghogomu, E.T., and Tugwell, P. Biologics for rheumatoid arthritis: an overview of Cochrane reviews. *Sao Paulo Med J*. 2010; 128:309-310.
69. Sakaguchi, N., Takahashi, T., Hata, H., Nomura, T., Tagami, T., Yamazaki, S., Sakihama, T., Matsutani, T., Negishi, I., Nakatsuru, S*., et al.* Altered thymic T-cell selection due to a mutation of the ZAP-70 gene causes autoimmune arthritis in mice. *Nature*. 2003; 426:454-460.
70. Chan, A.C., Iwashima, M., Turck, C.W., and Weiss, A. ZAP-70: a 70 kd protein-tyrosine kinase that associates with the TCR zeta chain. *Cell*. 1992; 71:649-662.
71. Yoshitomi, H., Sakaguchi, N., Kobayashi, K., Brown, G.D., Tagami, T., Sakihama, T., Hirota, K., Tanaka, S., Nomura, T., Miki, I*., et al.* A role for fungal {beta}-glucans and their receptor Dectin-1 in the induction of autoimmune arthritis in genetically susceptible mice. *J Exp Med*. 2005; 201:949-960.

72. Keffer, J., Probert, L., Cazlaris, H., Georgopoulos, S., Kaslaris, E., Kioussis, D., and Kollias, G. Transgenic mice expressing human tumour necrosis factor: a predictive genetic model of arthritis. *EMBO J*. 1991; 10:4025-4031.
73. Courtenay, J.S., Dallman, M.J., Dayan, A.D., Martin, A., and Mosedale, B. Immunisation against heterologous type II collagen induces arthritis in mice. *Nature*. 1980; 283:666-668.
74. Trentham, D.E., Townes, A.S., and Kang, A.H. Autoimmunity to type II collagen an experimental model of arthritis. *J Exp Med*. 1977; 146:857-868.
75. Wooley, P.H., Luthra, H.S., Stuart, J.M., and David, C.S. Type II collagen-induced arthritis in mice. I. Major histocompatibility complex (I region) linkage and antibody correlates. *J Exp Med*. 1981; 154:688-700.
76. Brunsberg, U., Gustafsson, K., Jansson, L., Michaëlsson, E., Ährlund-Richter, L., Pettersson, S., Mattsson, R., and Holmdahl, R. Expression of a transgenic class II Ab gene confers susceptibility to collagen-induced arthritis. *Eur J Immunol*. 1994; 24:1698-1702.
77. Kjellén, P., Brunsberg, U., Broddefalk, J., Hansen, B., Vestberg, M., Ivarsson, I., Engström, Å., Svejgaard, A., Kihlberg, J., Fugger, L.*, et al.* The structural basis of MHC control of collagen-induced arthritis; binding of the immunodominant type II collagen 256-270 glycopeptide to H-2Aq and H-2Ap molecules. *Eur J Immunol*. 1998; 28:755-767.
78. Holmdahl, R., Jansson, L., Andersson, M., and Larsson, E. Immunogenetics of type II collagen autoimmunity and susceptibility to collagen arthritis. *Immunology*. 1988; 65:305-310.
79. Holmdahl, R., Jonsson, R., Larsson, P., and Klareskog, L. Early appearance of activated CD4+ T lymphocytes and class II antigen-expressing cells in joints of DBA/1 mice immunized with type II collagen. *Lab Invest*. 1988; 58:53-60.
80. Michaelsson, E., Holmdahl, M., Engstrom, A., Burkhardt, H., Scheynius, A., and Holmdahl, R. Macrophages, but not dendritic cells, present collagen to T cells. *Eur J Immunol*. 1995; 25:2234-2241.
81. Corthay, A., Johansson, A., Vestberg, M., and Holmdahl, R. Collagen-induced arthritis development requires alpha beta T cells but not gamma delta T cells: studies with T cell-deficient (TCR mutant) mice. *Int Immunol*. 1999; 11:1065-1073.
82. Svensson, L., Jirholt, J., Holmdahl, R., and Jansson, L. B cell-deficient mice do not develop type II collagen-induced arthritis (CIA). *Clin Exp Immunol*. 1998; 111:521-526.
83. Michaelsson, E., Andersson, M., Engstrom, A., and Holmdahl, R. Identification of an immunodominant type-II collagen peptide recognized by T cells in H-2q mice: self tolerance at the level of determinant selection. *Eur J Immunol*. 1992; 22:1819-1825.
84. Stuart, J.M., and Dixon, F.J. Serum transfer of collagen-induced arthritis in mice. *J Exp Med*. 1983; 158:378-392.
85. Holmdahl, R., Rubin, K., Klareskog, L., Larsson, E., and Wigzell, H. Characterization of the antibody response in mice with type II collagen-induced arthritis, using monoclonal anti-type II collagen antibodies. *Arthritis Rheum*. 1986; 29:400-410.
86. Wang, Y., Rollins, S.A., Madri, J.A., and Matis, L.A. Anti-C5 monoclonal antibody therapy prevents collagen-induced arthritis and ameliorates established disease. *Proc Natl Acad Sci U S A*. 1995; 92:8955-8959.
87. Holmdahl, R., Jansson, L., Larsson, A., and Jonsson, R. Arthritis in DBA/1 mice induced with passively transferred type II collagen immune serum. Immunohistopathology and serum levels of anti-type II collagen auto-antibodies. *Scand J Immunol*. 1990; 31:147-157.
88. Wooley, P.H., Luthra, H.S., Singh, S.K., Huse, A.R., Stuart, J.M., and David, C.S. Passive transfer of arthritis to mice by injection of human anti-type II collagen antibody. *Mayo Clin Proc*. 1984; 59:737-743.
89. Terato, K., Hasty, K.A., Reife, R.A., Cremer, M.A., Kang, A.H., and Stuart, J.M. Induction of arthritis with monoclonal antibodies to collagen. *J Immunol*. 1992; 148:2103-2108.
90. Nandakumar, K.S., Svensson, L., and Holmdahl, R. Collagen type II-specific monoclonal antibody-induced arthritis in mice: description of the disease and the influence of age, sex, and genes. *Am J Pathol*. 2003; 163:1827-1837.

91. Terato, K., Harper, D.S., Griffiths, M.M., Hasty, D.L., Ye, X.J., Cremer, M.A., and Seyer, J.M. Collagen-induced arthritis in mice: synergistic effect of E. coli lipopolysaccharide bypasses epitope specificity in the induction of arthritis with monoclonal antibodies to type II collagen. *Autoimmunity*. 1995; 22:137-147.
92. Nandakumar, K.S., Backlund, J., Vestberg, M., and Holmdahl, R. Collagen type II (CII)-specific antibodies induce arthritis in the absence of T or B cells but the arthritis progression is enhanced by CII-reactive T cells. *Arthritis Res Ther*. 2004; 6:R544-550.
93. Hietala, M.A., Nandakumar, K.S., Persson, L., Fahlen, S., Holmdahl, R., and Pekna, M. Complement activation by both classical and alternative pathways is critical for the effector phase of arthritis. *Eur J Immunol*. 2004; 34:1208-1216.
94. Nandakumar, K.S., Andren, M., Martinsson, P., Bajtner, E., Hellstrom, S., Holmdahl, R., and Kleinau, S. Induction of arthritis by single monoclonal IgG anti-collagen type II antibodies and enhancement of arthritis in mice lacking inhibitory FcgammaRIIB. *Eur J Immunol*. 2003; 33:2269-2277.
95. Kouskoff, V., Korganow, A.S., Duchatelle, V., Degott, C., Benoist, C., and Mathis, D. Organ-specific disease provoked by systemic autoimmunity. *Cell*. 1996; 87:811-822.
96. Matsumoto, I., Staub, A., Benoist, C., and Mathis, D. Arthritis provoked by linked T and B cell recognition of a glycolytic enzyme. *Science*. 1999; 286:1732-1735.
97. Korganow, A.S., Ji, H., Mangialaio, S., Duchatelle, V., Pelanda, R., Martin, T., Degott, C., Kikutani, H., Rajewsky, K., Pasquali, J.L*., et al.* From systemic T cell self-reactivity to organ-specific autoimmune disease via immunoglobulins. *Immunity*. 1999; 10:451-461.
98. Maccioni, M., Zeder-Lutz, G., Huang, H., Ebel, C., Gerber, P., Hergueux, J., Marchal, P., Duchatelle, V., Degott, C., van Regenmortel, M*., et al.* Arthritogenic monoclonal antibodies from K/BxN mice. *J Exp Med*. 2002; 195:1071-1077.
99. Lee, D.M., Friend, D.S., Gurish, M.F., Benoist, C., Mathis, D., and Brenner, M.B. Mast cells: a cellular link between autoantibodies and inflammatory arthritis. *Science*. 2002; 297:1689-1692.
100. Wipke, B.T., and Allen, P.M. Essential role of neutrophils in the initiation and progression of a murine model of rheumatoid arthritis. *J Immunol*. 2001; 167:1601-1608.
101. Bruhns, P., Samuelsson, A., Pollard, J.W., and Ravetch, J.V. Colony-stimulating factor-1-dependent macrophages are responsible for IVIG protection in antibody-induced autoimmune disease. *Immunity*. 2003; 18:573-581.
102. Solomon, S., Rajasekaran, N., Jeisy-Walder, E., Snapper, S.B., and Illges, H. A crucial role for macrophages in the pathology of K/B x N serum-induced arthritis. *Eur J Immunol*. 2005; 35:3064-3073.
103. Ji, H., Ohmura, K., Mahmood, U., Lee, D.M., Hofhuis, F.M., Boackle, S.A., Takahashi, K., Holers, V.M., Walport, M., Gerard, C*., et al.* Arthritis critically dependent on innate immune system players. *Immunity*. 2002; 16:157-168.
104. Corr, M., and Crain, B. The role of FcgammaR signaling in the K/B x N serum transfer model of arthritis. *J Immunol*. 2002; 169:6604-6609.
105. Bockermann, R., Schubert, D., Kamradt, T., and Holmdahl, R. Induction of a B-cell-dependent chronic arthritis with glucose-6-phosphate isomerase. *Arthritis Res Ther*. 2005; 7:R1316-1324.
106. Schubert, D., Maier, B., Morawietz, L., Krenn, V., and Kamradt, T. Immunization with glucose-6-phosphate isomerase induces T cell-dependent peripheral polyarthritis in genetically unaltered mice. *J Immunol*. 2004; 172:4503-4509.
107. Frey, O., Bruns, L., Morawietz, L., Dunussi-Joannopoulos, K., and Kamradt, T. B cell depletion reduces the number of autoreactive T helper cells and prevents glucose-6-phosphate isomerase-induced arthritis. *PLoS One*. 2011; 6:e24718.
108. Frey, O., Reichel, A., Bonhagen, K., Morawietz, L., Rauchhaus, U., and Kamradt, T. Regulatory T cells control the transition from acute into chronic inflammation in glucose-6-phosphate isomerase-induced arthritis. *Ann Rheum Dis*69:1511-1518.
109. Matsumoto, I., Zhang, H., Yasukochi, T., Iwanami, K., Tanaka, Y., Inoue, A., Goto, D., Ito, S., Tsutsumi, A., and Sumida, T. Therapeutic effects of antibodies to tumor necrosis factor-alpha, interleukin-6 and cytotoxic T-lymphocyte antigen 4 immunoglobulin in mice with glucose-6-phosphate isomerase induced arthritis. *Arthritis Res Ther*. 2008; 10:R66.

110. Frey, O., Mitera, T., Kelchtermans, H., Schurgers, E., Kamradt, T., and Matthys, P. Ameliorated course of glucose-6-phosphate isomerase (G6PI)-induced arthritis in IFN-gamma receptor knockout mice exposes an arthritis-promoting role of IFN-gamma. *J Autoimmun*. 2011; 36:161-169.
111. Iwanami, K., Matsumoto, I., Tanaka, Y., Inoue, A., Goto, D., Ito, S., Tsutsumi, A., and Sumida, T. Arthritogenic T cell epitope in glucose-6-phosphate isomerase-induced arthritis. *Arthritis Res Ther*. 2008; 10:R130.
112. Bruns, L., Frey, O., Morawietz, L., Landgraf, C., Volkmer, R., and Kamradt, T. Immunization with an immunodominant self-peptide derived from glucose-6-phosphate isomerase induces arthritis in DBA/1 mice. *Arthritis Res Ther*. 2009; 11:R117.
113. Sareila, O., Kelkka, T., Pizzolla, A., Hultqvist, M., and Holmdahl, R. NOX2 Complex-derived ROS as Immune Regulators. *Antioxid Redox Signal*. 2011.
114. Dickinson, B.C., and Chang, C.J. Chemistry and biology of reactive oxygen species in signaling or stress responses. *Nat Chem Biol*. 2011; 7:504-511.
115. Gross, E., Sevier, C.S., Heldman, N., Vitu, E., Bentzur, M., Kaiser, C.A., Thorpe, C., and Fass, D. Generating disulfides enzymatically: reaction products and electron acceptors of the endoplasmic reticulum thiol oxidase Ero1p. *Proc Natl Acad Sci U S A*. 2006; 103:299-304.
116. Chen, K., Kirber, M.T., Xiao, H., Yang, Y., and Keaney, J.F., Jr. Regulation of ROS signal transduction by NADPH oxidase 4 localization. *J Cell Biol*. 2008; 181:1129-1139.
117. Bedard, K., and Krause, K.H. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol Rev*. 2007; 87:245-313.
118. Gelderman, K.A., Hultqvist, M., Pizzolla, A., Zhao, M., Nandakumar, K.S., Mattsson, R., and Holmdahl, R. Macrophages suppress T cell responses and arthritis development by producing Reactive Oxygen Species *J Clin Invest*. 2007; 117:3020-3028.
119. Kobayashi, S., Imajoh-Ohmi, S., Nakamura, M., and Kanegasaki, S. Occurrence of cytochrome b558 in B-cell lineage of human lymphocytes. *Blood*. 1990; 75:458-461.
120. Jackson, S.H., Devadas, S., Kwon, J., Pinto, L.A., and Williams, M.S. T cells express a phagocyte-type NADPH oxidase that is activated after T cell receptor stimulation. *Nat Immunol*. 2004; 5:818-827.
121. Babior, B.M., Curnutte, J.T., and Kipnes, B.S. Pyridine nucleotide-dependent superoxide production by a cell-free system from human granulocytes. *J Clin Invest*. 1975; 56:1035-1042.
122. Parkos, C.A., Allen, R.A., Cochrane, C.G., and Jesaitis, A.J. Purified cytochrome b from human granulocyte plasma membrane is comprised of two polypeptides with relative molecular weights of 91,000 and 22,000. *J Clin Invest*. 1987; 80:732-742.
123. Stasia, M.J., Bordigoni, P., Martel, C., and Morel, F. A novel and unusual case of chronic granulomatous disease in a child with a homozygous 36-bp deletion in the CYBA gene (A22(0)) leading to the activation of a cryptic splice site in intron 4. *Hum Genet*. 2002; 110:444-450.
124. Lambeth, J.D. NOX enzymes and the biology of reactive oxygen. *Nat Rev Immunol*. 2004; 4:181-189.
125. Johnson, J.L., Park, J.W., Benna, J.E., Faust, L.P., Inanami, O., and Babior, B.M. Activation of p47(PHOX), a cytosolic subunit of the leukocyte NADPH oxidase. Phosphorylation of ser-359 or ser-370 precedes phosphorylation at other sites and is required for activity. *J Biol Chem*. 1998; 273:35147-35152.
126. Sumimoto, H., Hata, K., Mizuki, K., Ito, T., Kage, Y., Sakaki, Y., Fukumaki, Y., Nakamura, M., and Takeshige, K. Assembly and activation of the phagocyte NADPH oxidase. Specific interaction of the N-terminal Src homology 3 domain of p47phox with p22phox is required for activation of the NADPH oxidase. *J Biol Chem*. 1996; 271:22152-22158.
127. Clark, R.A., Volpp, B.D., Leidal, K.G., and Nauseef, W.M. Two cytosolic components of the human neutrophil respiratory burst oxidase translocate to the plasma membrane during cell activation. *J Clin Invest*. 1990; 85:714-721.
128. Han, C.H., Freeman, J.L., Lee, T., Motalebi, S.A., and Lambeth, J.D. Regulation of the neutrophil respiratory burst oxidase. Identification of an activation domain in p67(phox). *J Biol Chem*. 1998; 273:16663-16668.

129. Takeya, R., Ueno, N., Kami, K., Taura, M., Kohjima, M., Izaki, T., Nunoi, H., and Sumimoto, H. Novel human homologues of p47phox and p67phox participate in activation of superoxide-producing NADPH oxidases. *J Biol Chem*. 2003; 278:25234-25246.
130. Wientjes, F.B., Hsuan, J.J., Totty, N.F., and Segal, A.W. p40phox, a third cytosolic component of the activation complex of the NADPH oxidase to contain src homology 3 domains. *Biochem J*. 1993; 296 (Pt 3):557-561.
131. Ellson, C.D., Davidson, K., Ferguson, G.J., O'Connor, R., Stephens, L.R., and Hawkins, P.T. Neutrophils from p40phox-/- mice exhibit severe defects in NADPH oxidase regulation and oxidant-dependent bacterial killing. *J Exp Med*. 2006; 203:1927-1937.
132. Suh, C.I., Stull, N.D., Li, X.J., Tian, W., Price, M.O., Grinstein, S., Yaffe, M.B., Atkinson, S., and Dinauer, M.C. The phosphoinositide-binding protein p40phox activates the NADPH oxidase during FcgammaIIA receptor-induced phagocytosis. *J Exp Med*. 2006; 203:1915-1925.
133. Diebold, B.A., and Bokoch, G.M. Molecular basis for Rac2 regulation of phagocyte NADPH oxidase. *Nat Immunol*. 2001; 2:211-215.
134. Koga, H., Terasawa, H., Nunoi, H., Takeshige, K., Inagaki, F., and Sumimoto, H. Tetratricopeptide repeat (TPR) motifs of p67(phox) participate in interaction with the small GTPase Rac and activation of the phagocyte NADPH oxidase. *J Biol Chem*. 1999; 274:25051-25060.
135. Kim, C., and Dinauer, M.C. Rac2 is an essential regulator of neutrophil nicotinamide adenine dinucleotide phosphate oxidase activation in response to specific signaling pathways. *J Immunol*. 2001; 166:1223-1232.
136. Abo, A., Pick, E., Hall, A., Totty, N., Teahan, C.G., and Segal, A.W. Activation of the NADPH oxidase involves the small GTP-binding protein p21rac1. *Nature*. 1991; 353:668-670.
137. Vaissiere, C., Le Cabec, V., and Maridonneau-Parini, I. NADPH oxidase is functionally assembled in specific granules during activation of human neutrophils. *J Leukoc Biol*. 1999; 65:629-634.
138. Borregaard, N., Heiple, J.M., Simons, E.R., and Clark, R.A. Subcellular localization of the b-cytochrome component of the human neutrophil microbicidal oxidase: translocation during activation. *J Cell Biol*. 1983; 97:52-61.
139. DeLeo, F.R., Renee, J., McCormick, S., Nakamura, M., Apicella, M., Weiss, J.P., and Nauseef, W.M. Neutrophils exposed to bacterial lipopolysaccharide upregulate NADPH oxidase assembly. *J Clin Invest*. 1998; 101:455-463.
140. Vulcano, M., Dusi, S., Lissandrini, D., Badolato, R., Mazzi, P., Riboldi, E., Borroni, E., Calleri, A., Donini, M., Plebani, A*., et al.* Toll receptor-mediated regulation of NADPH oxidase in human dendritic cells. *J Immunol*. 2004; 173:5749-5756.
141. Newburger, P.E., Ezekowitz, R.A., Whitney, C., Wright, J., and Orkin, S.H. Induction of phagocyte cytochrome b heavy chain gene expression by interferon gamma. *Proc Natl Acad Sci U S A*. 1988; 85:5215-5219.
142. Pithon-Curi, T.C., Levada, A.C., Lopes, L.R., Doi, S.Q., and Curi, R. Glutamine plays a role in superoxide production and the expression of p47phox, p22phox and gp91phox in rat neutrophils. *Clin Sci (Lond)*. 2002; 103:403-408.
143. Nunoi, H., Rotrosen, D., Gallin, J.I., and Malech, H.L. Two forms of autosomal chronic granulomatous disease lack distinct neutrophil cytosol factors. *Science*. 1988; 242:1298-1301.
144. Volpp, B.D., Nauseef, W.M., and Clark, R.A. Two cytosolic neutrophil oxidase components absent in autosomal chronic granulomatous disease. *Science*. 1988; 242:1295-1297.
145. Dusi, S., Donini, M., and Rossi, F. Mechanisms of NADPH oxidase activation: translocation of p40phox, Rac1 and Rac2 from the cytosol to the membranes in human neutrophils lacking p47phox or p67phox. *Biochem J*. 1996; 314 (Pt 2):409-412.
146. Leto, T.L., Adams, A.G., and de Mendez, I. Assembly of the phagocyte NADPH oxidase: binding of Src homology 3 domains to proline-rich targets. *Proc Natl Acad Sci U S A*. 1994; 91:10650-10654.
147. Inanami, O., Johnson, J.L., McAdara, J.K., Benna, J.E., Faust, L.R., Newburger, P.E., and Babior, B.M. Activation of the leukocyte NADPH oxidase by phorbol ester

requires the phosphorylation of p47PHOX on serine 303 or 304. *J Biol Chem*. 1998; 273:9539-9543.
148. DeSilva, U., Massa, H., Trask, B.J., and Green, E.D. Comparative mapping of the region of human chromosome 7 deleted in williams syndrome. *Genome Res*. 1999; 9:428-436.
149. Roesler, J., Curnutte, J.T., Rae, J., Barrett, D., Patino, P., Chanock, S.J., and Goerlach, A. Recombination events between the p47-phox gene and its highly homologous pseudogenes are the main cause of autosomal recessive chronic granulomatous disease. *Blood*. 2000; 95:2150-2156.
150. Francke, U., Ochs, H.D., Darras, B.T., and Swaroop, A. Origin of mutations in two families with X-linked chronic granulomatous disease. *Blood*. 1990; 76:602-606.
151. Olsson, L.M., Nerstedt, A., Lindqvist, A.K., Johansson, A.C., Medstrand, P., Olofsson, P., and Holmdahl, R. Copy Number Variation of the Gene NCF1 Is Associated with Rheumatoid Arthritis. *Antioxid Redox Signal*. 2011.
152. Green, S.P., Hamilton, J.A., Uhlinger, D.J., and Phillips, W.A. Expression of p47-phox and p67-phox proteins in murine bone marrow-derived macrophages: enhancement by lipopolysaccharide and tumor necrosis factor alpha but not colony stimulating factor 1. *J Leukoc Biol*. 1994; 55:530-535.
153. Rodaway, A.R., Teahan, C.G., Casimir, C.M., Segal, A.W., and Bentley, D.L. Characterization of the 47-kilodalton autosomal chronic granulomatous disease protein: tissue-specific expression and transcriptional control by retinoic acid. *Mol Cell Biol*. 1990; 10:5388-5396.
154. Segal, A.W., Jones, O.T., Webster, D., and Allison, A.C. Absence of a newly described cytochrome b from neutrophils of patients with chronic granulomatous disease. *Lancet*. 1978; 2:446-449.
155. Holmes, B., Page, A.R., and Good, R.A. Studies of the metabolic activity of leukocytes from patients with a genetic abnormality of phagocytic function. *J Clin Invest*. 1967; 46:1422-1432.
156. Quie, P.G., Kaplan, E.L., Page, A.R., Gruskay, F.L., and Malawista, S.E. Defective polymorphonuclear-leukocyte function and chronic granulomatous disease in two female children. *N Engl J Med*. 1968; 278:976-980.
157. Berendes, H., Bridges, R.A., and Good, R.A. A fatal granulomatosus of childhood: the clinical study of a new syndrome. *Minn Med*. 1957; 40:309-312.
158. van den Berg, J.M., van Koppen, E., Ahlin, A., Belohradsky, B.H., Bernatowska, E., Corbeel, L., Espanol, T., Fischer, A., Kurenko-Deptuch, M., Mouy, R.*, et al.* Chronic granulomatous disease: the European experience. *PLoS One*. 2009; 4:e5234.
159. Matute, J.D., Arias, A.A., Wright, N.A., Wrobel, I., Waterhouse, C.C., Li, X.J., Marchal, C.C., Stull, N.D., Lewis, D.B., Steele, M.*, et al.* A new genetic subgroup of chronic granulomatous disease with autosomal recessive mutations in p40 phox and selective defects in neutrophil NADPH oxidase activity. *Blood*. 2009; 114:3309-3315.
160. Jackson, S.H., Gallin, J.I., and Holland, S.M. The p47phox mouse knock-out model of chronic granulomatous disease. *J Exp Med*. 1995; 182:751-758.
161. Gerber, C.E., Bruchelt, G., Falk, U.B., Kimpfler, A., Hauschild, O., Kuci, S., Bachi, T., Niethammer, D., and Schubert, R. Reconstitution of bactericidal activity in chronic granulomatous disease cells by glucose-oxidase-containing liposomes. *Blood*. 2001; 98:3097-3105.
162. Segal, A.W., Geisow, M., Garcia, R., Harper, A., and Miller, R. The respiratory burst of phagocytic cells is associated with a rise in vacuolar pH. *Nature*. 1981; 290:406-409.
163. Reeves, E.P., Lu, H., Jacobs, H.L., Messina, C.G., Bolsover, S., Gabella, G., Potma, E.O., Warley, A., Roes, J., and Segal, A.W. Killing activity of neutrophils is mediated through activation of proteases by K+ flux. *Nature*. 2002; 416:291-297.
164. Kobayashi, S.D., Voyich, J.M., Braughton, K.R., Whitney, A.R., Nauseef, W.M., Malech, H.L., and DeLeo, F.R. Gene expression profiling provides insight into the pathophysiology of chronic granulomatous disease. *J Immunol*. 2004; 172:636-643.
165. Miller, J.L., Velmurugan, K., Cowan, M.J., and Briken, V. The type I NADH dehydrogenase of Mycobacterium tuberculosis counters phagosomal NOX2 activity to inhibit TNF-alpha-mediated host cell apoptosis. *PLoS Pathog*. 2010; 6:e1000864.
166. Brown, J.R., Goldblatt, D., Buddle, J., Morton, L., and Thrasher, A.J. Diminished production of anti-inflammatory mediators during neutrophil apoptosis and

macrophage phagocytosis in chronic granulomatous disease (CGD). *J Leukoc Biol*. 2003; 73:591-599.
167. Cedergren, J., Forslund, T., Sundqvist, T., and Skogh, T. Intracellular oxidative activation in synovial fluid neutrophils from patients with rheumatoid arthritis but not from other arthritis patients. *J Rheumatol*. 2007; 34:2162-2170.
168. Eggleton, P., Wang, L., Penhallow, J., Crawford, N., and Brown, K.A. Differences in oxidative response of subpopulations of neutrophils from healthy subjects and patients with rheumatoid arthritis. *Ann Rheum Dis*. 1995; 54:916-923.
169. El Benna, J., Hayem, G., Dang, P.M., Fay, M., Chollet-Martin, S., Elbim, C., Meyer, O., and Gougerot-Pocidalo, M.A. NADPH oxidase priming and p47phox phosphorylation in neutrophils from synovial fluid of patients with rheumatoid arthritis and spondylarthropathy. *Inflammation*. 2002; 26:273-278.
170. Seven, A., Guzel, S., Aslan, M., and Hamuryudan, V. Lipid, protein, DNA oxidation and antioxidant status in rheumatoid arthritis. *Clin Biochem*. 2008; 41:538-543.
171. Winkelstein, J.A., Marino, M.C., Johnston, R.B., Jr., Boyle, J., Curnutte, J., Gallin, J.I., Malech, H.L., Holland, S.M., Ochs, H., Quie, P*., et al.* Chronic granulomatous disease. Report on a national registry of 368 patients. *Medicine (Baltimore)*. 2000; 79:155-169.
172. Holland, S.M. Chronic granulomatous disease. *Clin Rev Allergy Immunol*. 2010; 38:3-10.
173. Pollock, J.D., Williams, D.A., Gifford, M.A., Li, L.L., Du, X., Fisherman, J., Orkin, S.H., Doerschuk, C.M., and Dinauer, M.C. Mouse model of X-linked chronic granulomatous disease, an inherited defect in phagocyte superoxide production. *Nat Genet*. 1995; 9:202-209.
174. Segal, B.H., Sakamoto, N., Patel, M., Maemura, K., Klein, A.S., Holland, S.M., and Bulkley, G.B. Xanthine oxidase contributes to host defense against Burkholderia cepacia in the p47(phox-/-) mouse model of chronic granulomatous disease. *Infect Immun*. 2000; 68:2374-2378.
175. Segal, B.H., Kuhns, D.B., Ding, L., Gallin, J.I., and Holland, S.M. Thioglycollate peritonitis in mice lacking C5, 5-lipoxygenase, or p47(phox): complement, leukotrienes, and reactive oxidants in acute inflammation. *J Leukoc Biol*. 2002; 71:410-416.
176. van de Loo, F.A., Bennink, M.B., Arntz, O.J., Smeets, R.L., Lubberts, E., Joosten, L.A., van Lent, P.L., Coenen-de Roo, C.J., Cuzzocrea, S., Segal, B.H*., et al.* Deficiency of NADPH oxidase components p47phox and gp91phox caused granulomatous synovitis and increased connective tissue destruction in experimental arthritis models. *Am J Pathol*. 2003; 163:1525-1537.
177. Qin, L., Liu, Y., Wang, T., Wei, S.J., Block, M.L., Wilson, B., Liu, B., and Hong, J.S. NADPH oxidase mediates lipopolysaccharide-induced neurotoxicity and proinflammatory gene expression in activated microglia. *J Biol Chem*. 2004; 279:1415-1421.
178. Olofsson, P., Nerstedt, A., Hultqvist, M., Nilsson, E.C., Andersson, S., Bergelin, A., and Holmdahl, R. Arthritis suppression by NADPH activation operates through an interferon-beta pathway. *BMC Biol*. 2007; 5:19.
179. Ha, H., and Lee, H.B. Reactive oxygen species amplify glucose signalling in renal cells cultured under high glucose and in diabetic kidney. *Nephrology (Carlton)*. 2005; 10 Suppl:S7-10.
180. Sun, Y., and Oberley, L.W. Redox regulation of transcriptional activators. *Free Radic Biol Med*. 1996; 21:335-348.
181. Bonizzi, G., Piette, J., Schoonbroodt, S., Greimers, R., Havard, L., Merville, M.P., and Bours, V. Reactive oxygen intermediate-dependent NF-kappaB activation by interleukin-1beta requires 5-lipoxygenase or NADPH oxidase activity. *Mol Cell Biol*. 1999; 19:1950-1960.
182. Khan, N., Ghousunnissa, S., Jegadeeswaran, S.M., Thiagarajan, D., Hasnain, S.E., and Mukhopadhyay, S. Anti-B7-1/B7-2 antibody elicits innate-effector responses in macrophages through NF-kappaB-dependent pathway. *Int Immunol*. 2007; 19:477-486.
183. Benito, M.J., Murphy, E., Murphy, E.P., van den Berg, W.B., FitzGerald, O., and Bresnihan, B. Increased synovial tissue NF-kappa B1 expression at sites adjacent to the cartilage-pannus junction in rheumatoid arthritis. *Arthritis Rheum*. 2004; 50:1781-1787.
184. Wang, Y., Zeigler, M.M., Lam, G.K., Hunter, M.G., Eubank, T.D., Khramtsov, V.V., Tridandapani, S., Sen, C.K., and Marsh, C.B. The role of the NADPH oxidase

complex, p38 MAPK, and Akt in regulating human monocyte/macrophage survival. *Am J Respir Cell Mol Biol*. 2007; 36:68-77.
185. Balce, D.R., Li, B., Allan, E.R., Rybicka, J.M., Krohn, R.M., and Yates, R.M. Alternative activation of macrophages by IL-4 enhances the proteolytic capacity of their phagosomes through synergistic mechanisms. *Blood*. 2011; 118:4199-4208.
186. Savina, A., Jancic, C., Hugues, S., Guermonprez, P., Vargas, P., Moura, I.C., Lennon-Dumenil, A.M., Seabra, M.C., Raposo, G., and Amigorena, S. NOX2 controls phagosomal pH to regulate antigen processing during crosspresentation by dendritic cells. *Cell*. 2006; 126:205-218.
187. Mantegazza, A.R., Savina, A., Vermeulen, M., Perez, L., Geffner, J., Hermine, O., Rosenzweig, S.D., Faure, F., and Amigorena, S. NADPH oxidase controls phagosomal pH and antigen cross-presentation in human dendritic cells. *Blood*. 2008; 112:4712-4722.
188. Rybicka, J.M., Balce, D.R., Khan, M.F., Krohn, R.M., and Yates, R.M. NADPH oxidase activity controls phagosomal proteolysis in macrophages through modulation of the lumenal redox environment of phagosomes. *Proc Natl Acad Sci U S A*. 2010; 107:10496-10501.
189. Bylund, J., MacDonald, K.L., Brown, K.L., Mydel, P., Collins, L.V., Hancock, R.E., and Speert, D.P. Enhanced inflammatory responses of chronic granulomatous disease leukocytes involve ROS-independent activation of NF-kappa B. *Eur J Immunol*. 2007; 37:1087-1096.
190. Warris, A., Netea, M.G., Wang, J.E., Gaustad, P., Kullberg, B.J., Verweij, P.E., and Abrahamsen, T.G. Cytokine release in healthy donors and patients with chronic granulomatous disease upon stimulation with Aspergillus fumigatus. *Scand J Infect Dis*. 2003; 35:482-487.
191. Hatanaka, E., Carvalho, B.T., Condino-Neto, A., and Campa, A. Hyperresponsiveness of neutrophils from gp 91phox deficient patients to lipopolysaccharide and serum amyloid A. *Immunol Lett*. 2004; 94:43-46.
192. Maly, F.E., Cross, A.R., Jones, O.T., Wolf-Vorbeck, G., Walker, C., Dahinden, C.A., and De Weck, A.L. The superoxide generating system of B cell lines. Structural homology with the phagocytic oxidase and triggering via surface Ig. *J Immunol*. 1988; 140:2334-2339.
193. Vene, R., Delfino, L., Castellani, P., Balza, E., Bertolotti, M., Sitia, R., and Rubartelli, A. Redox remodeling allows and controls B-cell activation and differentiation. *Antioxid Redox Signal*. 2010; 13:1145-1155.
194. Gruhne, B., Sompallae, R., Marescotti, D., Kamranvar, S.A., Gastaldello, S., and Masucci, M.G. The Epstein-Barr virus nuclear antigen-1 promotes genomic instability via induction of reactive oxygen species. *Proc Natl Acad Sci U S A*. 2009; 106:2313-2318.
195. Corzo, C.A., Cotter, M.J., Cheng, P., Cheng, F., Kusmartsev, S., Sotomayor, E., Padhya, T., McCaffrey, T.V., McCaffrey, J.C., and Gabrilovich, D.I. Mechanism regulating reactive oxygen species in tumor-induced myeloid-derived suppressor cells. *J Immunol*. 2009; 182:5693-5701.
196. Tacke, R., Lee, H.C., Goh, C., Courtney, J., Polyak, S.J., Rosen, H.R., and Hahn, Y.S. Myeloid suppressor cells induced by hepatitis C virus suppress T cell responses through the production of reactive oxygen species. *Hepatology*. 2011.
197. Gelderman, K.A., Hultqvist, M., Holmberg, J., Olofsson, P., and Holmdahl, R. T cell surface redox levels determine T cell reactivity and arthritis susceptibility. *Proc Natl Acad Sci U S A*. 2006; 103:12831-12836.
198. Efimova, O., Szankasi, P., and Kelley, T.W. Ncf1 (p47phox) is essential for direct regulatory T cell mediated suppression of CD4+ effector T cells. *PLoS One*. 2011; 6:e16013.
199. Mizuki, K., Kadomatsu, K., Hata, K., Ito, T., Fan, Q.W., Kage, Y., Fukumaki, Y., Sakaki, Y., Takeshige, K., and Sumimoto, H. Functional modules and expression of mouse p40(phox) and p67(phox), SH3-domain-containing proteins involved in the phagocyte NADPH oxidase complex. *Eur J Biochem*. 1998; 251:573-582.
200. Williams, M.S., and Kwon, J. T cell receptor stimulation, reactive oxygen species, and cell signaling. *Free Radic Biol Med*. 2004; 37:1144-1151.
201. Ling, S., Li, Z., Borschukova, O., Xiao, L., Pumpens, P., and Holoshitz, J. The rheumatoid arthritis shared epitope increases cellular susceptibility to oxidative stress

by antagonizing an adenosine-mediated anti-oxidative pathway. *Arthritis Res Ther*. 2007; 9:R5.
202. Hultqvist, M., Olofsson, P., Gelderman, K.A., Holmberg, J., and Holmdahl, R. A new arthritis therapy with oxidative burst inducers. *PLoS Med*. 2006; 3:e348.
203. Denu, J.M., and Tanner, K.G. Specific and reversible inactivation of protein tyrosine phosphatases by hydrogen peroxide: evidence for a sulfenic acid intermediate and implications for redox regulation. *Biochemistry*. 1998; 37:5633-5642.
204. Salmeen, A., and Barford, D. Functions and mechanisms of redox regulation of cysteine-based phosphatases. *Antioxid Redox Signal*. 2005; 7:560-577.
205. Nakashima, I., Kato, M., Akhand, A.A., Suzuki, H., Takeda, K., Hossain, K., and Kawamoto, Y. Redox-linked signal transduction pathways for protein tyrosine kinase activation. *Antioxid Redox Signal*. 2002; 4:517-531.
206. Gringhuis, S.I., Papendrecht-van der Voort, E.A., Leow, A., Nivine Levarht, E.W., Breedveld, F.C., and Verweij, C.L. Effect of redox balance alterations on cellular localization of LAT and downstream T-cell receptor signaling pathways. *Mol Cell Biol*. 2002; 22:400-411.
207. Gringhuis, S.I., Leow, A., Papendrecht-Van Der Voort, E.A., Remans, P.H., Breedveld, F.C., and Verweij, C.L. Displacement of linker for activation of T cells from the plasma membrane due to redox balance alterations results in hyporesponsiveness of synovial fluid T lymphocytes in rheumatoid arthritis. *J Immunol*. 2000; 164:2170-2179.
208. Romagnoli, P., Strahan, D., Pelosi, M., Cantagrel, A., and van Meerwijk, J.P. A potential role for protein tyrosine kinase p56(lck) in rheumatoid arthritis synovial fluid T lymphocyte hyporesponsiveness. *Int Immunol*. 2001; 13:305-312.
209. Romani, L., Fallarino, F., De Luca, A., Montagnoli, C., D'Angelo, C., Zelante, T., Vacca, C., Bistoni, F., Fioretti, M.C., Grohmann, U*., et al.* Defective tryptophan catabolism underlies inflammation in mouse chronic granulomatous disease. *Nature*. 2008; 451:211-215.
210. De Ravin, S.S., Zarember, K.A., Long-Priel, D., Chan, K.C., Fox, S.D., Gallin, J.I., Kuhns, D.B., and Malech, H.L. Tryptophan/kynurenine metabolism in human leukocytes is independent of superoxide and is fully maintained in chronic granulomatous disease. *Blood*. 2010; 116:1755-1760.
211. George-Chandy, A., Nordstrom, I., Nygren, E., Jonsson, I.M., Postigo, J., Collins, L.V., and Eriksson, K. Th17 development and autoimmune arthritis in the absence of reactive oxygen species. *Eur J Immunol*. 2008; 38:1118-1126.
212. Hagenow, K., Gelderman, K.A., Hultqvist, M., Merky, P., Backlund, J., Frey, O., Kamradt, T., and Holmdahl, R. Ncf1-associated reduced oxidative burst promotes IL-33R+ T cell-mediated adjuvant-free arthritis in mice. *J Immunol*. 2009; 183:874-881.
213. Hultqvist, M., Sareila, O., Vilhardt, F., Norin, U., Olsson, L.M., Olofsson, P., Hellman, U., and Holmdahl, R. Positioning of a polymorphic quantitative trait nucleotide in the Ncf1 gene controlling oxidative burst response and arthritis severity in rats. *Antioxid Redox Signal*. 2011; 14:2373-2383.
214. Kumar Shakya, A., Kumar, A., Klaczkowska, D., Hultqvist, M., Hagenow, K., Holmdahl, R., and Nandakumar, K.S. A Collagen and a Thermo-Responsive Polymer of N-Isopropylacrylamide Induces Arthritis Independent of Toll-Like Receptors: A Strong Influence by Major Histocompatibility Complex Class II and Ncf1 Genes. *Am J Pathol*. 2011.
215. Batsalova, T., Dzhambazov, B., Klaczkowska, D., and Holmdahl, R. Mice producing less reactive oxygen species are relatively resistant to collagen glycopeptide vaccination against arthritis. *J Immunol*. 2010; 185:2701-2709.
216. Hultqvist, M., Backlund, J., Bauer, K., Gelderman, K.A., and Holmdahl, R. Lack of Reactive Oxygen Species Breaks T Cell Tolerance to Collagen Type II and Allows Development of Arthritis in Mice. *J Immunol*. 2007; 179:1431-1437.
217. Batsalova, T., Dzhambazov, B., Merky, P., Backlund, A., and Backlund, J. Breaking T cell tolerance against self type II collagen in HLA-DR4-transgenic mice and development of autoimmune arthritis. *Arthritis Rheum*62:1911-1920.
218. Mossberg, N., Andersen, O., Nordin, M., Nilsson, S., Svedhem, A., Bergstrom, T., Hellstrand, K., and Movitz, C. Leukocyte oxygen radical production determines disease severity in the recurrent Guillain-Barre syndrome. *J Inflamm (Lond)*. 2010; 7:40.

219. Mossberg, N., Andersen, O., Nilsson, S., Dahlgren, C., Hellstrand, K., Lindh, M., Svedhem, A., Bergstrom, T., and Movitz, C. Oxygen radical production and severity of the Guillain--Barre syndrome. *J Neuroimmunol*. 2007; 192:186-191.
220. Mossberg, N., Movitz, C., Hellstrand, K., Bergstrom, T., Nilsson, S., and Andersen, O. Oxygen radical production in leukocytes and disease severity in multiple sclerosis. *J Neuroimmunol*. 2009; 213:131-134.
221. Kobayashi, S., Imajoh-Ohmi, S., Kuribayashi, F., Nunoi, H., Nakamura, M., and Kanegasaki, S. Characterization of the superoxide-generating system in human peripheral lymphocytes and lymphoid cell lines. *J Biochem*. 1995; 117:758-765.
222. Zhou, H., Duncan, R.F., Robison, T.W., Gao, L., and Forman, H.J. Ca(2+)-dependent p47phox translocation in hydroperoxide modulation of the alveolar macrophage respiratory burst. *Am J Physiol*. 1997; 273:L1042-1047.
223. Gough, P.J., Gordon, S., and Greaves, D.R. The use of human CD68 transcriptional regulatory sequences to direct high-level expression of class A scavenger receptor in macrophages in vitro and in vivo. *Immunology*. 2001; 103:351-361.